Characterization of mechanisms of antibiotic resistance in *Neisseria gonorrhoeae*

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Abstract

MARGARET ELIZABETH DUNCAN: Characterization of mechanisms of antibiotic resistance in *Neisseria gonorrhoeae*
(Under the direction of Robert A. Nicholas, PhD)

Neisseria gonorrhoeae is a Gram-negative diplococcus that causes the sexually transmitted infection, gonorrhea. As a naturally competent organism, *N. gonorrhoeae* can take up genetic material from outside the cell and incorporate it into its own DNA through homologous recombination. This has allowed the pathogen to generate antigenic diversity for escape from the human immune system and to develop and spread antibiotic resistance genes. This mode of resistance, chromosomally mediated resistance, is complex and requires at least five resistance determinants. Four of these determinants have been identified at the molecular level: *penA* (mutations in penicillin-binding protein 2 [PBP 2]), *mtrR* (overexpression of the MtrC-MtrD-MtrE efflux pump), *penB* (mutations in $PorB_{1b}$), and *ponA* (mutation in PBP 1). These determinants can be readily transferred from a penicillin-resistant donor (FA6140) to a susceptible recipient strain (FA19). However, despite repeated attempts, transformation to high-level penicillin resistance equivalent to the donor strain has not been achieved.

I initiated studies to further elucidate the complex mechanisms of chromosomally mediated antibiotic resistance in *N. gonorrhoeae*. First, I characterized unique mutations

in *mtrR* and *penB* found in a group of clinical isolates from New Caledonia. These mutations proved to have weaker phenotypes than the more common mutations. I also identified a set of 67 genes found only in penicillin-resistant strains of *N. gonorrhoeae*. When transformed into a penicillin-sensitive strain, none of the genes increased resistance. Finally, I initiated studies to elucidate the mechanisms of high-level penicillin resistance and to characterize "Factor X", the unknown gene(s) that is (are) responsible for high-level resistance. My results indicate that the phenotype of Factor X is independent of the other four resistance determinants and is expressed phenotypically even in the absence of other determinants. Additionally, Factor X plays a role in increasing resistance to bactericidal, but not bacteriostatic, antibiotics, reminiscent of studies in *E. coli* whereby bactericidal antibiotics, no matter what pathway they inhibit, kill bacteria through a common oxidative stress mechanism. Together, the results of these studies add to our knowledge about the interactions between *N. gonorrhoeae* and antimicrobials, and further our understanding of resistance mechanisms.

To my dad, who encouraged me and believed in me, even when I didn't believe in myself. Thank you for not letting me give up and for reminding me that science and learning are fun.

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List of Abbreviations

ASPG-R	asialoglycoprotein receptor
BLAST	Basic Local Alignment Search Tool
CDC	Centers for Disease Control
CEACAM	carcinoembryonic antigen cell adhesion molecules
CFU	colony forming units
CR3	complement receptor 3
CO_2	carbon dioxide
DGI	disseminated gonococcal infection
fH	factor H
GC	gonococcus/gonococcal
GCB	gonococcal medium base
GISP	gonococcal isolate surveillance project
hcG	human chorionic gonadotropin
HIV	human immunodeficiency virus
HSPG	heparin sulfate proteoglycans
HV	hypervariable
LHr	lutropin receptor
LOS	lipooligosaccharide
LPS	lipopolysaccharide
MIC	minimum inhibitory concentration
NCBI	National Center for Biotechnology Information
OD	optical density

ORF	open reading frame
Opa	opacity associated protein
PBP	penicillin binding protein
PCR	polymerase chain reaction
PIA	porin IA
PIB	porin IB
PID	pelvic inflammatory disease
PMN	polymorphonuclear leukocyte
qRT-PCR	quantitative real-time polymerase chain reaction
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
STI	sexually transmitted infection
Tfp	Type IV pilus
TNF-α	tumor necrosis factor alpha
US	uptake sequence
WHO	World Health Organization

Chapter 1

Introduction

1.1 Introduction

Neisseria gonorrhoeae is the causative agent of the sexually transmitted infection, gonorrhea. Gonococcal infections are the second most commonly reported sexually transmitted infections to the Centers for Disease Control, with over 300,000 cases reported in 2010. The bacterium infects both men and women, usually causing localized infections of the genital tract (McCormack, Stumacher et al. 1977; Sherrard and Barlow 1996). If left untreated, it can lead to more serious complications including pelvic inflammatory disease, ectopic pregnancy, and infertility in women, as well as disseminated gonococcal infections in both sexes (Paavonen 1998; Bleich, Sheffield et al. 2012). *N. gonorrhoeae* is an obligate human pathogen that has developed multiple mechanisms to escape the immune system and resist killing by antimicrobial agents. Resistance to nearly all classes of antibiotics has developed, making infections with *N. gonorrhoeae* increasingly difficult to treat.

1.2 Epidemiology

The Gonococcal Isolate Surveillance Project (GISP) is the CDC program designed to collect data regarding numbers of infections and antibiotic susceptibility of

Neisseria gonorrhoeae isolates in the United States (Schwarcz, Zenilman et al. 1990). In 2010, there were 309,341 cases of gonorrhea reported to the CDC. For the first time since 2006, this represented an increase in the rates of infection. Rates of infection are the highest among adolescents and young adults, minorities, and women (Figure 1.1). Southern states have the highest rates of infection, consistent with reports from previous years (Figure 1.2). The increase in infections in 2010 was broad; nearly all regions of the country reported more gonococcal infections, with only the Midwest reporting a decrease. There were more men and women of all races/ethnicities infected. The highest increase was seen among adults aged 20-24. Because females with gonorrhea are often asymptomatic, they can be unknowing carriers of the bacteria (males can also be asymptomatic, but this occurs at a much lower rate than females). Thus estimates of the actual disease burden may be up to twice as high as the reported number.

Estimating the number of worldwide gonococcal infections is a challenging task. In 2005, the World Health Organization estimated that there were nearly 95 million new cases of gonorrhea, with the majority of cases occurring in Southeast Asia and Sub-Saharan Africa (World Health Organization 2009). The numbers of cases reported in the 2005 study are much higher than the 1995 and 1999 WHO estimates (Table 1.1) (World Health Organization 2001). While some of the increases are due to increased rates of infection, officials also believe that the 2005 estimates are more accurate due to better data collection between 2000-2005. The 1995 and 1999 values were most likely very conservative estimates.

Figure 1.1 *N. gonorrhoeae* infections among various age groups, gender, and race

Bar graphs representing the rates of infection among age groups, gender, and races in the United States, 2010. Rates of infection are the highest among young adults, women, and blacks. Infection rates per 100,000 people are shown.

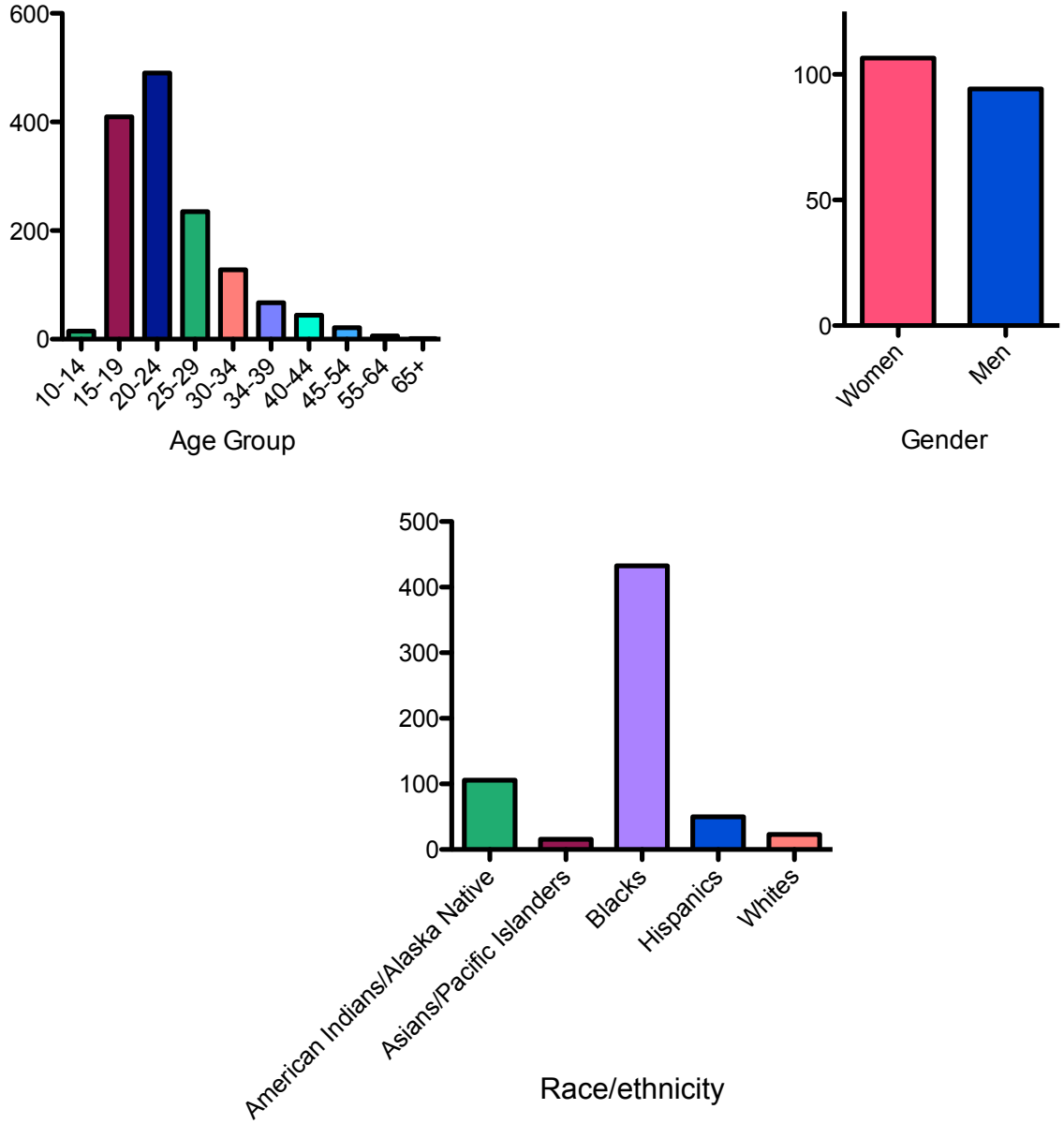
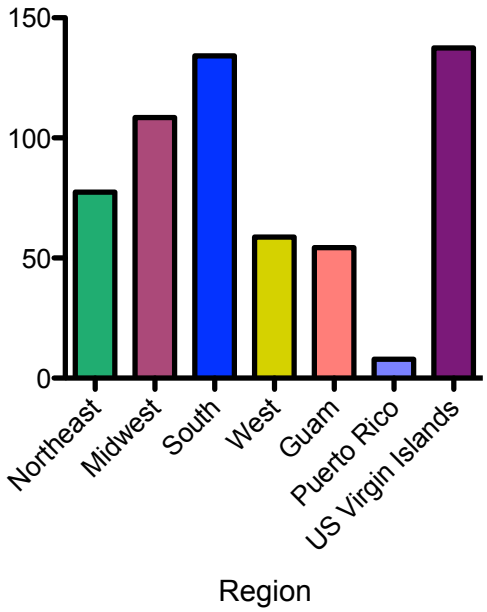


Figure 1.2 Geographical distribution of gonorrhea infections across the United States

Bar graph showing rates of infection (per 100,000 people) across the United States. The map shows states with higher rates of infection in darker colors. (Map adapted from (Centers for Disease Control and Prevention 2011)



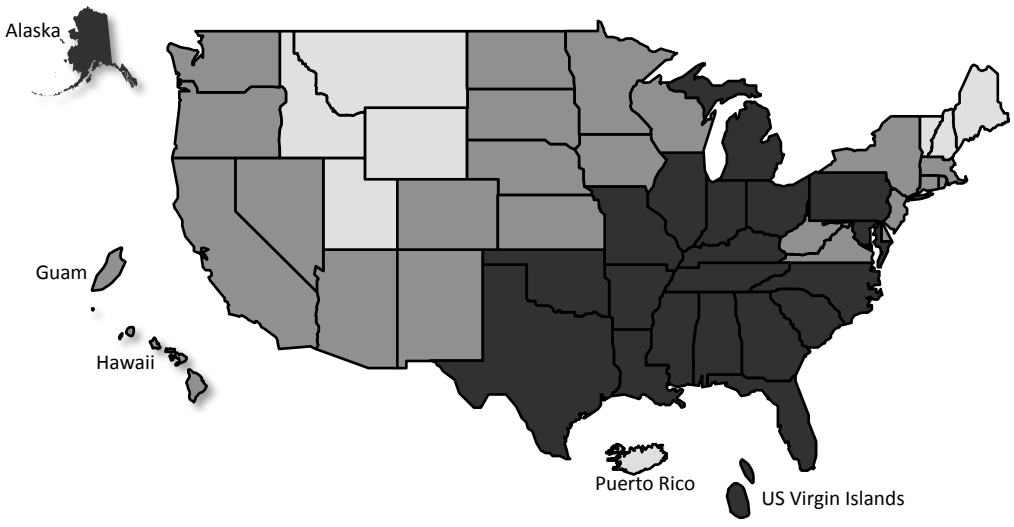


Table 1.1 WHO estimation of gonorrhea infections worldwide, 1995, 1999 and 2005.

Table comparing data collected by the World Health Organization in 1995, 1999, and 2005. Estimated numbers (in millions) of gonococcal infections in different regions worldwide are shown. Numbers of infections in 2005 are significantly higher, perhaps because of more accurate measurements as well as an increase in the rates of infections (World Health Organization 2001; World Health Organization 2009).

Region	**1995**	**1995**	**2005**	**Region**
North America	1.75	1.56	0.7	North America
Latin America and Caribbean	7.12	7.27	8.8	Latin America and Caribbean
Europe and Central Asia	3.55	4.42	12	Europe and Central Asia
South and Southeast Asia	29.11	27.20	22.7	South and Southeast Asia
East Asia and Australia/New Zealand	3.40	3.39	26.9	East Asia and Pacific
North Africa and Middle East	1.54	1.47	6.5	North Africa and Middle East
Sub-Saharan Africa	15.67	17.03	17.3	Sub-Saharan Africa
Total	**62.14**	**62.34**	**94.9**	**Total**

1.3 Clinical Manifestations

Infections with *N. gonorrhoeae* are typically localized to mucosa of the genital tract, but they can cause serious complications in other body parts. Mucosal infections can also occur in other areas.

1.3.1 Genital infections

Men infected with *N. gonorrhoeae* typically show symptoms of urethritis. The majority of men report urethral discharge only, while others suffer from dysuria or a combination of both symptoms (Spence 1983) Interestingly, patients co-infected with *Chlamydia trachomatis* are less likely to display symptoms than those infected with *N. gonorrhoeae* alone (Sherrard and Barlow 1996; Bozicevic, Fenton et al. 2006). Because the two bacteria are often found in the same populations, individuals infected are at risk for being undiagnosed and further spreading disease. The incubation period for urethral *N. gonorrhoeae* in the male is approximately one week, though a small number (<10%) of men with gonococcal urethritis remain asymptomatic (Handsfield, Lipman et al. 1974; McNagny, Parker et al. 1992; Sherrard 1996). Because they have no symptoms, these men do not seek treatment and the bacterium persists, putting sexual partners at risk for infection.

Localized complications of male urethral gonorrhea can occur. The most common complication is epididymitis, with testicular pain and swelling as the predominant symptoms (Berger, Alexander et al. 1979; Holmes, Berger et al. 1979). The periurethral glands can become infected, resulting in abscesses and penile masses (Fan 2010; Kenfak-Foguena, Zarkik et al. 2010; Parashar and Schechtman 2011). General penile edema is

another possible complication. Other rare complications include urethral stricture, acute and chronic prostatitis, perineal swelling from infection of Cowper's glands, seminal vesiculitis, urethral fistulae, and penile sclerosing lymphangitis (Pandhi and Reddy 2002; Rosen and Hwong 2003; Marrazzo 2009; Parashar and Schechtman 2011).

Women infected with *N. gonorrhoeae* are often asymptomatic or do not seek treatment for symptoms (Platt, Rice et al. 1983). The endocervical canal is often infected and abnormal, purulent vaginal discharge may be the only presenting symptom (McCormack, Stumacher et al. 1977). Other symptoms might include dysuria and abnormal vaginal bleeding (Brunham, Paavonen et al. 1984). Symptoms usually develop within 3-10 days of exposure, or not at all (Nolan and Osborne 1973; McCormack, Stumacher et al. 1977; Platt, Rice et al. 1983). In fact, up to 80% of women infected with gonorrhea remain asymptomatic or do not seek treatment for their mild, non-specific symptoms (Pedersen and Bonin 1971; Nolan and Osborne 1973; McCormack, Stumacher et al. 1977; Spence 1983). These women remain at risk for further complications and also pose a public health risk as carriers of the bacterium.

As with men, the female urethra and periurethral glands may become infected. Complications include abscesses of Bartholin's glands (Nolan and Osborne 1973). By far the most common sequela is ascending infection, resulting in Pelvic Inflammatory Disease (Swasdio, Rugpao et al. 1996).

1.3.2 Anorectal infections

Anorectal gonorrhea infections are possible in both men and women, either through direct sexual contact or contamination from secretions from a urethral or cervical

infection. As with other gonococcal infections, anorectal infections are often asymptomatic (Quinn, Stamm et al. 1983; Turner, Rogers et al. 2002). When symptoms are present, anal pruritus, tenesmus, discharge, constipation, and bleeding are the common complaints (Miller 2006). Those infected through direct anal intercourse are more likely to develop symptoms (Marcus, Bernstein et al. 2010).

1.3.3 Pelvic inflammatory disease

Pelvic inflammatory disease (PID) can develop if cervical infection with *N. gonorrhoeae* is not identified and treated. *N. gonorrhoeae* and *Chlamydia trachomatis* are the most common pathogens associated with PID, though respiratory pathogens and endogenous bacteria from the vagina and colon have also been implicated in PID pathogenesis (McCormack, Nowroozi et al. 1977; Soper 1994; Ness, Kip et al. 2005). It is estimated that PID occurs in approximately 10-40% of women with gonorrhea (Paavonen 1998; Walker and Sweet 2011). Vaginal douching greatly increases the likelihood of the development of PID (Wolner-Hanssen, Eschenbach et al. 1990; Scholes, Daling et al. 1993; Ness, Hillier et al. 2005). PID may be asymptomatic for months or years in up to 85% of cases, leading to increased chances of further complications (Hillis, Joesoef et al. 1993; Walker and Sweet 2011). As the pathogen ascends from the cervix into the uterus and fallopian tubes, acute salpingitis and endometritis can ensue (Holmes, Eschenbach et al. 1980; Westrom 1980). Other possible clinical manifestations include tubo-ovarian abscesses and pelvic peritonitis. Bilateral pelvic pain, fever, discharge, and bleeding are possible symptoms. Low abdominal pain is common, with or without signs of a lower genital tract infection (Peipert and Soper 1994). Perihepatitis may develop if

the liver capsule is exposed to *N. gonorrhoeae* from the fallopian tubes (Lopez-Zeno, Keith et al. 1985). Damage to the fallopian tubes often occurs, causing infertility in approximately 15-20% of patients with PID (Westrom, Joesoef et al. 1992). Multiple cases of PID significantly increase the chance of infertility (Paavonen 1998). Tubal scarring leads to ectopic pregnancy in 9% of patients with PID, to chronic pelvic pain in 27% (Westrom, Joesoef et al. 1992; Haggerty, Schulz et al. 2003; Walker and Sweet 2011).

1.3.4 Infections of the oro-pharynx

Neisseria gonorrhoeae can colonize the oro-pharynx but is usually either mild or asymptomatic. In fact, less than 10% of those infected seek treatment for symptoms (Morris, Klausner et al. 2006). When symptoms are present, a sore throat and dysphagia are the complaints (Wiesner, Tronca et al. 1973; Tice and Rodriguez 1981). There may be pharyngeal exudates and associated lymphadenopathy on the physical exam. Oro-pharyngeal infection is most often, but not always, accompanied by a co-existing urogenital infection (Gunn, O'Brien et al. 2008)

Because symptoms are usually absent, those infected with pharyngeal *N. gonorrhoeae* are possible carriers of the pathogen. As many as 5% of homosexual men are infected with pharyngeal gonorrhea (Morris, Klausner et al. 2006). These silent pharyngeal infections can serve as a source for urogenital and anorectal infections (Kinghorn 2010). Pharyngeal gonorrhea is also a potential source for disseminated gonococcal infection in the host.

1.3.5 Disseminated gonococcal infection

Disseminated gonococcal infection is a rare complication of gonorrhea. In approximately 0.5-3% of cases, *N. gonorrhoeae* spreads from the mucosal surface to other body parts, usually the skin and joints (Holmes, Counts et al. 1971; Kerle, Mascola et al. 1992). The arthritis-dermatitis syndrome that is most common begins with joint pain that involves the knees and elbows, though it may affect other joints (Guillot, Delattre et al. 2012). A characteristic skin rash develops with pustules on the extremities (Nolan and Osborne 1973). Finally, septic arthritis of one joint develops (Rice 2005). As mentioned above, the pharynx is a possible source of the pathogen in cases of DGI (Wiesner, Tronca et al. 1973). Thus the patient may or may not have first developed symptoms of a urogenital infection. Women are more likely to be affected by DGI, and the risk of dissemination is highest after menstruation and while pregnant (O'Brien, Goldenberg et al. 1983; Burgis and Nawaz 2006). Patients with deficiencies in the complement cascade pathway are also at an increased risk for DGI (Ellison, Curd et al. 1987). DGI during pregnancy increases the risks of miscarriage, preterm labor, and perinatal morbidity and mortality (Burgis and Nawaz 2006; Bleich, Sheffield et al. 2012).

While the arthritis-dermatitis syndrome is the most common manifestation of DGI, other more serious conditions are possible. Bacterial endocarditis is a rare complication, usually involving the aortic valve (50%) or mitral valve (30%) (Jackman and Glamann 1991; Akkinepally, Douglass et al. 2010). Ascending aortitis and aortic aneurysm have also been reported (Woo, Rabkin et al. 2011). Other possible fatal manifestations of DGI are gonococcal meningitis, septicemia, and respiratory distress

syndrome (Holmes, Counts et al. 1971; Handsfield 1975; Belding and Carbone 1991; Billings, Evans et al. 1991; Thiery, Tankovic et al. 2001).

1.3.6 Primary cutaneous infection

While rare, it is possible to acquire a gonococcal infection of the skin, even without any mucosal surface being infected. These infections are typically found in the genital region or on the finger and occur through inoculation of a preexisting lesion. They vary in appearance, from pyogenic granulomas to pustules, and can result in localized cellulitis (Scott and Scott 1982; Ghosn and Kibbi 2004).

1.3.7 Ocular infection

Infection of the eye with *N. gonorrhoeae* causes conjunctivitis. It is uncommon and usually results from autoinoculation, but can also be a result of direct inoculation from a sexual partner. Occupationally acquired gonococcal conjunctivitis has been reported in laboratory workers (Dillard 2011) Community outbreaks of gonococcal conjunctivitis have been reported in Australia and Africa (Mikru, Molla et al. 1991; Merianos, Condon et al. 1995; Matters, Wong et al. 1998; Mak, Smith et al. 2001). In a 1997 outbreak in Australia, the strain involved in the eye infections was not the same strain causing genital infections in the region, indicating that factors other than auto-inoculation or sexual contact were involved in the spread of the disease (Mak, Smith et al. 2001). Mucopurulent exudate, photophobia, and pain are characteristic of the infection (Day, Ramkissoon et al. 2006). Because the pathogen is able to successfully penetrate the

corneal epithelium, the condition can rapidly progress and result in keratoconjunctivitis, with subsequent corneal ulceration and even corneal perforation (Tjia, van Putten et al. 1988). If treatment is not sought quickly, blindness is likely (Tong, Tan et al. 2004; Tipple, Smith et al. 2010).

1.3.8 Neonatal infections

Pregnant women infected with *N. gonorrhoeae* can transmit the infection to neonates during delivery (Cates and Alexander 1988). During delivery, the infant is exposed to both the infected cervix and also directly to the bacterium (Shore and Winkelstein 1971). Transmission typically results in gonococcal conjunctivitis (Woods 2005). Pneumonia, arthritis, pharyngitis, and rectal infections are also possible but not as common (Gregory, Chisom et al. 1972; Handsfield, Hodson et al. 1973; Alexander 1988). Gonococcal scalp abscesses have been reported in newborns at the site of placement of intrauterine fetal scalp electrodes (Plavidal and Werch 1977; Reveri and Krishnamurthy 1979). Because the immune system is not fully developed in newborns, infections can progress rapidly from direct inoculation of bacteria into sub-cutaneous tissue. If not treated, the newborn can develop serious complications such as osteomyelitis, meningitis, or septicemia (Asnis and Brennessel 1992). Because of increased prenatal care, including screening for *N. gonorrhoeae* and the widespread routine use of prophylaxis for newborn gonococcal conjunctivitis, neonatal gonococcal infections are far less common today than in the past (Hammerschlag, Cummings et al. 1989; Laga, Meheus et al. 1989; Zuppa, D'Andrea et al. 2011).

1.4 History of gonorrhea

Neisseria gonorrhoeae is an obligate human pathogen. In fact, there is evidence suggesting that the pathogen has been infecting humans for thousands of years. Hippocrates described infections resembling male gonococcal urethritis and ancient Chinese writings have been recovered describing similar. Several books of the Old Testament, including Genesis, Leviticus, and Numbers, describe a condition that is likely gonorrhea. Interestingly, the Greek physician Galen gave the disease the name gonorrhea ("flow of seed") in 130AD, but the disease was often confused with syphilis until much later. In 1879, microbiologist Albert Neisser definitively identified the causative organism, *Neisseria gonorrhoeae*.

Effective therapeutics were not available until the mid-20th century, when the sulfonamides were introduced (Cokkinis 1937). Earlier treatment attempts included the application of heat, "anti-toxins", vaccines made up of killed gonococci, mercury, irrigation, catheterization, electrolysis, suction, and ingestion of systemic purgatives (Broughton 1821; Anonymous 1845; M'Vail D 1884; Stockman 1911; Cambell 1916; Russ 1917; Statham 1928; Kanner 1932; Burke, Gabe et al. 1938).

1.5 Transmission and public health recommendations

The transmission efficiency of gonorrhea depends on the anatomic site of infection and on the type of contact between infected individuals. Male-to-female transmission is likely more efficient than female-to-male, resulting in a risk of approximately 50-70% per sexual episode (Lin, Donegan et al. 1998). The risk of a woman passing the infection to a male urethra during vaginal intercourse is

approximately 20% per sexual episode. However, this risk increases to 60-80% after four or more exposures (Holmes, Johnson et al. 1970; Hooper, Reynolds et al. 1978). Studies have shown that gonorrhea may be passed to the male urethra from the pharynx during fellatio, but the rate of transmission is unknown (Tice and Rodriguez 1981; Lafferty, Hughes et al. 1997; Janier, Lassau et al. 2003; Bernstein, Stephens et al. 2009). Anal transmission is likely efficient, though this has not been quantified.

Individuals with significant symptoms of disease are most likely to seek treatment for infection, limiting their ability to spread the bacterium. As mentioned above though, up to 10% of men and 80% of women do not display signs of early genital infection, allowing them to serve as reservoirs of infection for the sexually active population. Screening of sexually active young women at annual exams has helped to bring down the numbers of gonococcal infections in the United States since the mid-1970's. Men who have sex with men (MSM) should also be screened for rectal and pharyngeal infection, as these sites are often asymptomatic but serve as sources for genital infections.

1.5.1 Gonorrhea and other STIs

Individuals infected with *N. gonorrhoeae* are at risk for other sexually transmitted infections (STIs). The CDC recommends that all people being treated for gonorrhea also receive treatment for *Chlamydia trachomatis* infections (Marrazzo 2009). In 2000, the CDC found that nearly half of all 15-19 year olds infected and almost 40 percent of 20-24 year olds with gonorrhea were also infected with chlamydia (Dicker, Mosure et al. 2003). A similar study among men in Italy showed that nearly half of the men diagnosed with gonococcal urethritis were also infected with *C. trachomatis* (Donati, Di Francesco et al.

2009). In 2005, a study showed a high number of cases of concurrent gonorrhea and trichomonas, gonorrhea and chlamydia, or gonorrhea, trichomonas, and chlamydia (Khan, Fortenberry et al. 2005). A recent study among men in India found a strong association between infection with gonorrhea and syphilis, as well as weaker associations between gonorrhea and *Candida albicans,* chancroid, and herpes. Women in the same study had co-infections of *N. gonorrhoeae* and *C. albicans, C. trachomatis,* or *Trichomonas vaginalis* (Bala, Mullick et al. 2011*)*. While the rates of co-infection with specific pathogens vary among locations, it is clear that infection with *N. gonorrhoeae* increases the chances that a patient will be infected with another STI. This is one of the reasons that gonococcal infections must be diagnosed and treated with effective antibiotics.

1.5.2 *N. gonorrhoeae* and HIV

An additional that *N. gonorrhoeae* poses a public health risk is because individuals infected with the bacterium have an increased likelihood of both transmitting and acquiring human immunodeficiency virus (HIV). Individuals infected with both gonorrhea and HIV experience increased viral shedding because of increased localized expression of the virus (Chen, Boulton et al. 2003). Treatment of gonococcal urethritis causes a decrease in HIV viral concentration in semen (Cohen, Hoffman et al. 1997). Exposure of primary resting CD4(+) T cells to *N. gonorrhoeae* increases the likelihood that these cells will be infected by HIV-1 (Ding, Rapista et al. 2010). Clearly, the synergy between HIV and *N. gonorrhoeae* is an important public health issue and several problematic scenarios are apparent: 1) an individual is infected with gonorrhea and

exposed to HIV (increased likelihood of acquiring HIV). 2) An individual infected with HIV and exposed to gonorrhea (increased likelihood of acquiring gonorrhea) 3) An individual infected with both gonorrhea and HIV exposes another person to both pathogens (partner is at risk for both HIV and gonorrhea).

1.6 Biology of the gonococcus

N. gonorrhoeae belongs to the genus *Neisseria*, which includes 12 species that colonize human mucosal surfaces. The only other pathogenic species in the genus is *Neisseria meningitidis*, the causative agent of bacterial meningitis. *N. gonorrhoeae* is an obligate human pathogen that has evolved with man over thousands of years.

N. gonorrhoeae is a gram-negative diplococcal organism. Strict growth requirements in culture include a temperature of 35-37°C and approximately 5% atmospheric CO_2 (Spence, Wright et al. 2008). Bicarbonate can be substituted for CO_2 (Morse and Bartenstein 1974). The bacterium thrives in a humid environment. *N. gonorrhoeae* typically grows aerobically but can also grow anaerobically if nitrite is provided (Knapp and Clark 1984). Because the organism has complex nutritional needs, special media have been developed for growing it in the laboratory. *N. gonorrhoeae* requires iron for growth and is usually able to extract it from the human host; therefore iron must be supplemented in the culture medium. Media must also be supplemented with glucose, lactate, or pyruvate as these provide a carbon source for the pathogen (Catlin 1973). Kellogg's Supplements are often added to the media to meet the nutritional requirements (Kellogg, Peacock et al. 1963). Colonies appear on agar plates within 12-48 hours, but viability decreases dramatically after 48 hours. Growth in liquid culture is

difficult. The doubling time is approximately one hour in liquid, but cells have a tendency to die for no apparent reason.

Four different colony morphologies are noticeable on agar plates. As detailed below, *N. gonorrhoeae* expresses opacity proteins (Opa) and a type IV pilus (Tfp) in a phase variable manner. Thus at any given time, colonies may be piliated (P+) and may appear opaque in color (Opa+). Colony types 1 and 2 are small, shiny, and convex due to the expression of the pilus in these types. Piliated colonies may spontaneously lose the pili and appear dull and flat, as in colony types 3 or 4 (Kellogg, Peacock et al. 1963). Colonies expressing opacity proteins (Opa+) appear yellowish and darker, while Opa- colonies are translucent (Walstad, Guymon et al. 1977).

1.6.1 Structure

N. gonorrhoeae has the structure of a typical gram-negative bacterium. The cell wall is composed of an outer membrane and an inner membrane that are separated by a thin layer of peptidoglycan. The outer membrane contains several proteins, phospholipids, and lipooligosaccharide, all of which are important for the biology of the organism.

1.6.1.1 Lipooligosaccharide

Lipooligosaccharide (LOS) is an important component of the outer membrane of *N. gonorrhoeae* and contributes to the virulence of the bacterium. All gram-negative organisms express lipopolysaccharides (LPS). These glycolipids help to maintain the structure of the outer membrane and also serve as virulence factors. The structure of LPS

consists of a Lipid A moiety to serve as an anchor to the membrane, a core oligosaccharide, and a polymer of a repeating polysaccharide. In contrast, the LOS in *Neisseria* species contains a truncated non-repeating oligosaccharide in place of the repeating polysaccharide. (Figure 1.3) (Griffiss, Schneider et al. 1988; Banerjee, Wang et al. 1998).

The structure of LOS expressed by the gonococcus is variable. During infection, different sugars are attached to the core, depending on which LOS biosynthetic proteins are expressed (Yang and Gotschlich 1996). The length and composition of the side chains varies widely, because the LOS biosynthetic enzymes are highly phase variable (Mandrell, Schneider et al. 1986; Griffiss, O'Brien et al. 1987; Gibson, Webb et al. 1989; Gibson, Melaugh et al. 1993; Yamasaki, Kerwood et al. 1994). The family of genes that transfer sugar residues, the *lgt* genes, are highly phase variable. Single nucleotide insertions within the poly-C or poly-G repeats in the *lgt* genes are a result of slip-strand mispairing during replication (Gotschlich 1994; Danaher, Levin et al. 1995; Yang and Gotschlich 1996; Banerjee, Wang et al. 1998). This allows the genes to be turned "on" or "off" quickly, ultimately resulting in various side chains attaching to the LOS core. Additionally, several promoters are found within the cluster of genes that encode for the glycosyltransferases, contributing to further variation in the LOS expression at any time (Braun and Stein 2004). The side changes can undergo spontaneous conversion at any time when the cell begins to express different glycosyltransferases, allowing a single cell to alter its LOS production. Hybrid genes are created by recombination of the different glycosyltransferase genes, contributing to antigenic diversity (Tong, Arking et al. 2002). Additional variation occurs upon sialyation of the LOS core sugars (Kim, Zhou et al.

1992; van Putten and Robertson 1995). Sialyation of LOS contributes to the ability of the bacterium to evade immune system defenses (Schneider, Griffiss et al. 1985; Smith, Parsons et al. 1995).

1.6.1.2 Opa

Opacity associated proteins (Opa) are another set of highly variable proteins found in the outer membrane of *N. gonorrhoeae* (Swanson 1978; Swanson 1978; Lambden, Heckels et al. 1979). Gonococci typically encode the information for 11 Opa proteins but only express up to 3 at a time (Black, Schwalbe et al. 1984; Connell, Shaffer et al. 1990). Expression of the different Opa proteins occurs at the translational level, by frame-shifts that occur among the *opa* genes due to varying numbers of repeats of the leader sequence (Stern, Brown et al. 1986).The strength of the promoter also contributes to phase variation (Belland, Morrison et al. 1997). Hypervariable (HV) regions of the *opa* genes contribute to the antigeneic diversity seen among expressed Opa proteins (Connell, Shaffer et al. 1990). Differential expression of Opa proteins can be found among isolates of the same strain.

Opa proteins can be divided into two groups, depending on the human cellular receptor to which they bind (Dehio, Gray-Owen et al. 1998). Most Opa proteins interact with the carcinoembryonic antigen-related family of cell adhesion molecules (CEACAM) (Wang, Gray-Owen et al. 1998). The other class of Opa proteins binds host cell heparin sulfate proteoglycans (HSPG) (van Putten, Duensing et al. 1998).

Opa proteins are made up of 8 antiparallel β-strands that form a barrel in the outer membrane. This part of the protein remains relatively conserved throughout the family.

Four extracellular loops link the β-strands of the barrel together, and loops 2 and 3 contain the HV regions of the protein (Sadarangani, Pollard et al. 2011). Opa^+ bacterial cells are almost always present in natural gonococcal infection. The only time that mainly Opa^- cells are recovered is during female menses (James and Swanson 1978; Swanson, Barrera et al. 1988; Jerse, Cohen et al. 1994).

1.6.1.3 Pili

Type IV pili (Tfp) are found protruding from the outer membrane of *N. gonorrhoeae*. Tfp are relatively conserved in different gram-negative bacteria and are important for pathogenesis, motility, and transformation (Strom and Lory 1993; Craig, Pique et al. 2004). Several different proteins are associated with Tfp structure and biogenesis. The pilus itself is a helical structure comprised primarily of the major pilin, PilE. Five PilE subunits are arranged into three distinct layers of the pilus: a highly conserved core, a less-conserved central layer, and a hypervariable outer layer (Swanson 1973; Schoolnik, Fernandez et al. 1984; So, Billyard et al. 1985). The structure of the pilus reveals a core made up of the N-terminus ends of the subunits, organized into a helix of overlapping α-helices. The amino terminal of pilE is highly conserved among different pilins (Schoolnik, Fernandez et al. 1984). Each pilin subunit has a 4-stranded antiparallel β-sheet and a sugar loop. The 4-stranded antiparallel β-sheet and sugar loop from each subunit are held together by hydrogen bonds to form a continuous β-sheet that makes up the central layer of the pilus fiber. The outermost region of the pilus has a β-hairpin and the C-terminal tail. The outer layer is poorly conserved and is flexible to

allow for a large variety of amino acids, contributing to the antigenic variation among *N. gonorrhoeae* pilins (Forest and Tainer 1997).

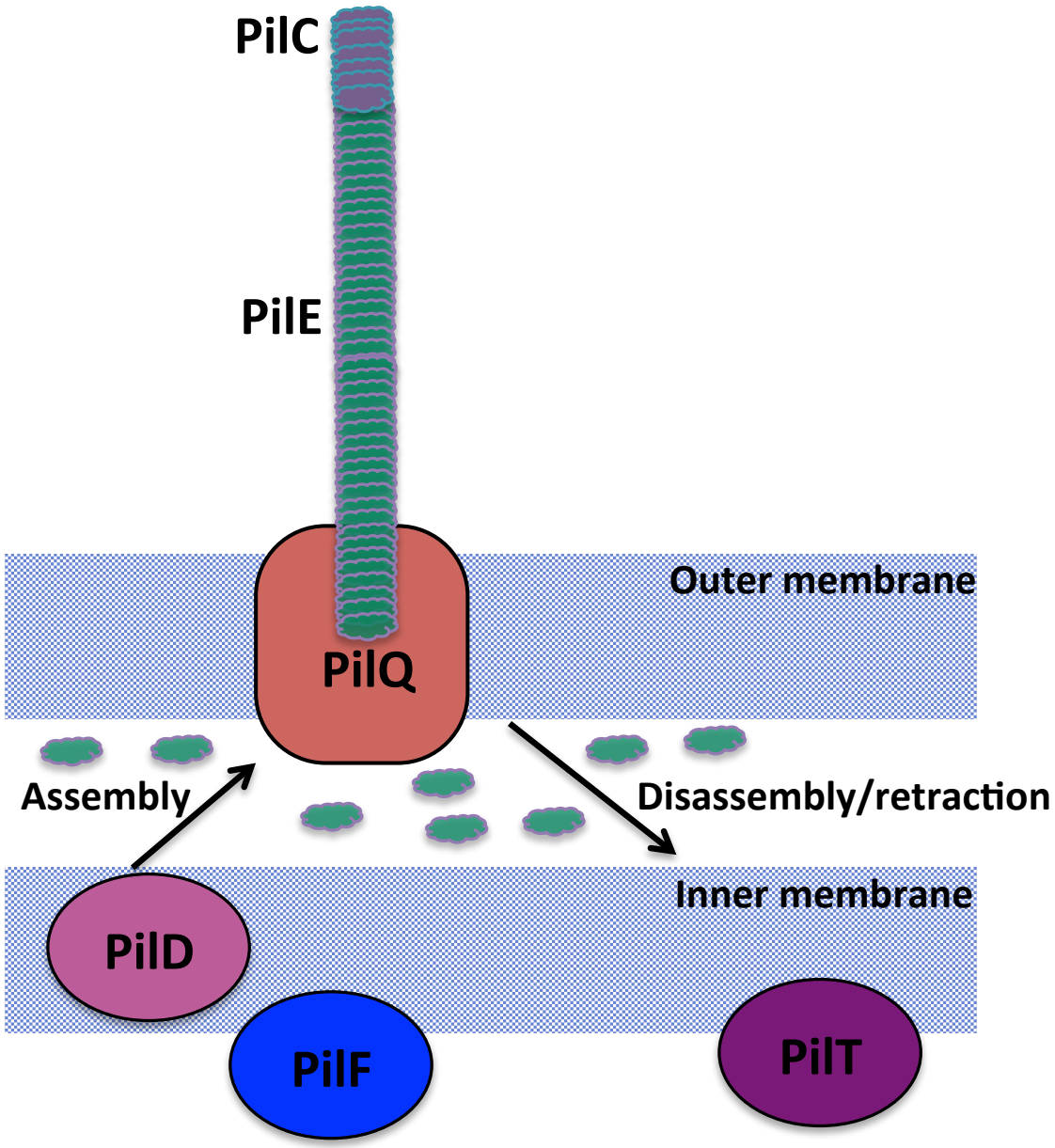
The pilus extends beyond the outer membrane, passing through the membrane protein PilQ (Figure 1.4) (Chen, Tobiason et al. 2004). Retraction of the pilus back through PilQ and into the cell contributes to the twitching motion seen among piliated gonococci. Expression of several proteins is involved in pilus formation and pilus-associated functions (Meyer, Billyard et al. 1984; Drake and Koomey 1995; Wolfgang, Park et al. 1998; Wolfgang, van Putten et al. 2000; Helm, Barnhart et al. 2007). Among others, two ATPases, PilF and PilT are involved in pilus assembly and retraction, respectively (Freitag, Seifert et al. 1995; Wolfgang, Lauer et al. 1998; Forest, Satyshur et al. 2004). PilV is associated with adhesion to host epithelial cells (Winther-Larsen, Hegge et al. 2001) and PilD is involved with pilin processing and translocation to the membrane surface (Koomey, Bergstrom et al. 1991; Freitag, Seifert et al. 1995). Evidence suggests that tip of the pilus is composed of PilC, the pilus adhesion protein (Jonsson, Nyberg et al. 1991; Rudel, Scheurerpflug et al. 1995).

PilE undergoes both phase and antigenic variation. Regions of silent loci, *pilS,* may be spontaneously recombined at the expression locus, *pilE*, allowing for the expression of antigenically variable pili (Meyer, Mlawer et al. 1982; Meyer, Billyard et al. 1984; Haas and Meyer 1986; Segal, Hagblom et al. 1986). The regions of *pilS* are donated to *pilE* in a non-reciprocal manner; thus the original *pilS* sequence does not change (Howell-Adams, Wainwright et al. 1996; Howell-Adams and Seifert 2000). Homologous recombination with DNA from other gonococcal cells also contributes to the variability among pilins (Serkin and Seifert 1998).These features allow for extreme

variation among pili, such that almost all cells express a different pilus. Different sequences of pilE can be found in isolates from the same strain and different pilins are expressed by the gonococcus during the course of infection (Hagblom, Segal et al. 1985; Seifert, Wright et al. 1994). The pilus also undergoes phase variation. After approximately 24 hours of growth in vitro, piliated colonies shift to become non-piliated. A deletion in the expression site typically causes the P^+ to P^- shift (Segal, Billyard et al. 1985; Seifert 1996)

Figure 1.3 Type IV pilus structure and formation

The figure shows the basic structure of the *N. gonorrhoeae* type IV pilus. PilC is the adhesin located at the tip of the main pilus. The main pilus is made up of five units of PilE, which is translocated through the secretin PilQ. PilF and PilD are involved in pilin processing and formation while PilT is involved in pilin retraction. (Figure adapted from (Wolfgang, van Putten et al. 2000).



1.6.1.4 Porin

Porins (PorB), which are water-filled channels that traverse the outer membrane of *N. gonorrhoeae*, are the most abundant protein in the outer membrane. This hydrophilic channel is a trimer of three identical subunits. Together, the subunits form a β-pleated-barrel structure. The barrel functions as a channel across the hydrophobic membrane, allowing the flow of ions and other small molecules between the bacterium and the environment (Young, Blake et al. 1983; Nikaido 1994; Achouak, Heulin et al. 2001). PorB also contributes to the pathogenicity of *N. gonorrhoeae* (Blake and Gotschlich 1983).

N. gonorrhoeae PorB can be found in one of two classes, $PorB_{1A}$ (PIA) or $PorB_{1B}$ (PIB) (Knapp, Tam et al. 1984). The alleles for both PIA and PIB are found at the same locus and each strain of *N. gonorrhoeae* expresses only one of the two subclasses, though PIA/PIB hybrids are possible (Gotschlich, Seiff et al. 1987; Carbonetti, Simnad et al. 1988; Gill, Jayamohan et al. 1994; Cooke, Jolley et al. 1998). There are significant differences between PIA and PIB. The two classes have different orientations in the outer membrane. P1A is oriented such that a short portion of the N-terminus exposed to the environment, whereas the both termini of P1B are embedded in the outer membrane and the central portion is exposed to the extracellular environment (Judd 1989). Genetic variation is found among the exposed portions of both subclasses of PorB, a characteristic that is used in subtyping strains.

Both forms of porin contribute to the pathogenicity of *N. gonorrhoeae*. PorB can translocate into the eukaryotic cell membrane, inhibit phagosome maturation, assist with bacterial entry into host cells, and down-regulate cell surface receptors necessary for

immune function (Lynch, Blake et al. 1984; Rudel, Schmid et al. 1996; Mosleh, Huber et al. 1998; van Putten, Duensing et al. 1998; Bauer, Rudel et al. 1999).

Of the two subclasses of porin, $PorB_{1A}$ is more commonly found in disseminated gonococcal infections (Cannon, Buchanan et al. 1983). PIA is associated with mediating serum resistance, which the bacterium to escape normal human immune system responses (Ram, McQuillen et al. 1998; Ram, Cullinane et al. 2001). PIA is also better at facilitating host cell invasion than PIB (Garvin, Bash et al. 2008). PIB, on the other hand, is usually associated with localized infections and contributes to anti-microbial resistance. Isolates expressing PIB exhibit higher baseline resistance to antibiotics and mutations in this allele that contribute to antibiotic resistance have been described (Bygdeman, Mardh et al. 1984; Carbonetti, Simnad et al. 1990; Gill, Simjee et al. 1998; Olesky, Hobbs et al. 2002).

1.6.1.5 Peptidoglycan

N. gonorrhoeae has a thin layer of peptidoglycan separating the inner and outer membranes. The peptidoglycan layer of the cell represents < 2% of the total mass of the dry cell (Hebeler and Young 1976). Glycan chains are connected by short peptides to form a mesh-like structure that provides strength and support to the cell wall. Fragments of peptidoglycan released during cell wall synthesis are also linked to the pathogenesis of *N. gonorrhoeae*. The glycan chains are made up of alternating residues of *N*-acetyl glucosamine and *N*-acetylmuramic acid, which are connected by a β-(1,4) linkage. A peptide chain composed of L-Ala-D-γ-Glu-*m*-DAP-D-Ala-D-Ala is attached to each *N*-acetylmuramic acid residue (Hebeler and Young 1976). Transpeptidation of the peptide

chains creates the cross-linked glycan chains with a ridged, strong structure. Penicillin-binding proteins (PBPs) recognize the D-Ala-D-Ala end of the peptide chains and catalyze the release of the terminal D-Ala. (Ishino, Mitsui et al. 1980; Barbour 1981) The *m*-DAP on a neighboring peptide chain can then interact with the D -Ala to form a cross-link (Rosenthal, Wright et al. 1980). The glycan chains in *N. gonorrhoeae* range between 80-110 disaccharides long (Hebeler and Young 1976).

During cellular growth, fragments of peptidoglycan are released into the environment. Lytic transglycosylases cleave the bonds between the *N*-acetyl glucosamine and *N*-acetylmuramic acid residues and catalyze the formation of a 1,6-anhydro bond on the *N*-acteylmuramic acid (Rosenthal 1979; Cloud and Dillard 2002; Cloud-Hansen, Hackett et al. 2008). The peptidoglycan monomers that are released are cytotoxic to human cells, and the release of these monomers has been linked to fallopian cell damage and the processes of disseminated gonococcal infection (Melly, McGee et al. 1984; Cloud-Hansen, Hackett et al. 2008).

1.7 Biology of infection

1.7.1 Adherence to human cells

Several of the structures mentioned above play an important role in *N. gonorrhoeae* attachment to human cells. Pili, Opa, LOS, and porin are all involved in the association between bacterial and human cells.

Pili were implicated as adhesions in early pathogenesis studies. The pilus is necessary for the initial attachment to cells and the establishment of sustained infections (Punsalang and Sawyer 1973; Swanson 1973; McGee, Johnson et al. 1981; Merz and So

2000). Pili are important for attachment to host epithelial cells. PilC, located at the tip of the pilus, functions as an adhesin (Rudel, Scheurerpflug et al. 1995). Several studies have shown that CD46 (complement regulatory protein) serves as a receptor for the *N. gonorrhoeae* pilus to bind to the human cell (Kallstrom, Liszewski et al. 1997; Kallstrom, Blackmer Gill et al. 2001). However, there is also evidence of CD46-independent pilus binding (Kirchner, Heuer et al. 2005). In cervical epithelial cells, CR3 appears to be the primary receptor for the gonococcal pilus. Interestingly, the binding of pilus to CR3 is also dependent on porin (Edwards, Brown et al. 2002). During infection of the male urethra, the pilus binds to $\alpha_1\beta_1$ or $\alpha_2\beta_1$ integrins and the gonococcus-integrin complex next binds the asialoglycoprotein receptor (ASGP-R) on urethral epithelial cells (Edwards and Apicella 2005). Lipooligosaccharide then associates with the ASGP-R, creating a tight bond between the bacterium and the epithelium (Harvey, Porat et al. 2000; Harvey, Jennings et al. 2001).

The gonococcal pilus might contribute to ascending infection of the upper female genital tract. Type IV pili are capable of causing a "twitching" motility (Merz, So et al. 2000; Mattick 2002). This motility might aid the bacterium in ascending infection. In the upper female genital tract, the primary receptor for gonococcal adherence appears to be the lutropin receptor (LHr) along endometrial and fallopian tube epithelial cells (Spence, Chen et al. 1997). A gonococcal surface ribosomal protein, L12, mimics hCG, the normal human ligand for the LHr (Cruz, Anderson et al. 1987; Spence and Clark 2000; Ascoli, Fanelli et al. 2002).

Opa proteins are also involved in binding to both epithelial cells and immune system cells. Opa proteins bind to one of two categories of receptors: carcinoembryonic

antigen cell adhesion molecules (CEACAMS) or heparin sulphate proteoglycans (HSPGs). Different types of CEACAMs are found on a variety of human cells. Epithelial cells express CEACAM1, CEACAM6, and CEACAM6 and Opa-CEACAM binding facilitates gonococcal adherence to epithelial cells (Wang, Gray-Owen et al. 1998; Sadarangani, Pollard et al. 2011). CEACAM3 is the primary CEACAM found on neutrophils, though CEACAM1 and CEACAM6 are also present at smaller levels. Opa-CEACAM attachments on neutrophils are important for the bacterium to attach to the neutrophil, signaling downstream events related to invasion and killing (Chen and Gotschlich 1996; Gray-Owen, Dehio et al. 1997; Popp, Dehio et al. 1999; Chen, Bolland et al. 2001). Interactions between *N. gonorrhoeae* Opa and CEACAMs found on both T and B lymphocytes might also contribute to the pathogenicity of the bacterium by down-regulating the immune response (Boulton and Gray-Owen 2002; Sadarangani, Pollard et al. 2011)

While pili seem to provide the initial attachment to human cells, Opa interactions with receptors on the cells are necessary for internalization of the bacterium (Dehio, Gomez-Duarte et al. 1998). On epithelial cells, Opa-HSPG binding triggers transcytosis of the bacteria into the host cell (Grassme, Ireland et al. 1996; Grassme, Gulbins et al. 1997). Opa attachment to CEACAMs on neutrophils can trigger phagocytosis of the bacterium and also stimulates the oxidative burst of the neutrophils (Gray-Owen, Dehio et al. 1997; Hauck, Meyer et al. 1998).

Lipooligosaccharide molecules on the bacterial surface are also important for the initial attachment to host cells. As discussed earlier, the oligosaccharide units attached to the LOS core are highly variable. The structure of these chains can mimic human

glycosphingolipds, allowing the LOS molecules to bind host receptors that are meant to associate with the human glycosphingolipids (Harvey, Swords et al. 2001). Bacterial cells that do not express Opa utilize LOS to attach to host cells (Schneider, Cross et al. 1995; Song, Ma et al. 2000). On male urethral epithelial cells, LOS interactions with ASGP-R provide a tight connection between the bacterium and the host.

1.7.2 Invasion of human cells and induction of the inflammatory response

As discussed in section 1.7.1, several surface molecules of *N. gonorrhoeae* are involved with the initial attachment to host cells. These same molecules are also linked to host cell invasion by the bacterium. Pili and porin are necessary for the gonococcus to bind to CR3 on cervical epithelial cells. That interaction activates a signaling cascade, resulting in ruffling of the epithelial cell to engulf the bacterium (Griffiss, Lammel et al. 1999; Merz, Enns et al. 1999). In the cells of the upper female genital tract, attachment of the bacteria to non-ciliated epithelium promotes sloughing of the nearby ciliated epithelial cells (McGee, Stephens et al. 1983; Stephens, McGee et al. 1987). Trancytosis through the non-ciliated epithelial cell, as well as the sloughing of the ciliated epithelium, provide the bacterial cells with access to subepithelial tissues (McGee, Stephens et al. 1983).

The inflammatory response to gonococcal infections results from interactions between bacterial virulence factors and host cells. Nearly all men with gonorrhea are symptomatic, due to the influx of neutrophils and the release of inflammatory cytokines near the urethral epithelium. LOS causes urethral epithelial cells to secrete TNF-α, IL-1β, IL-6, and IL-8 (Ramsey, Schneider et al. 1995; Harvey, Post et al. 2002). These

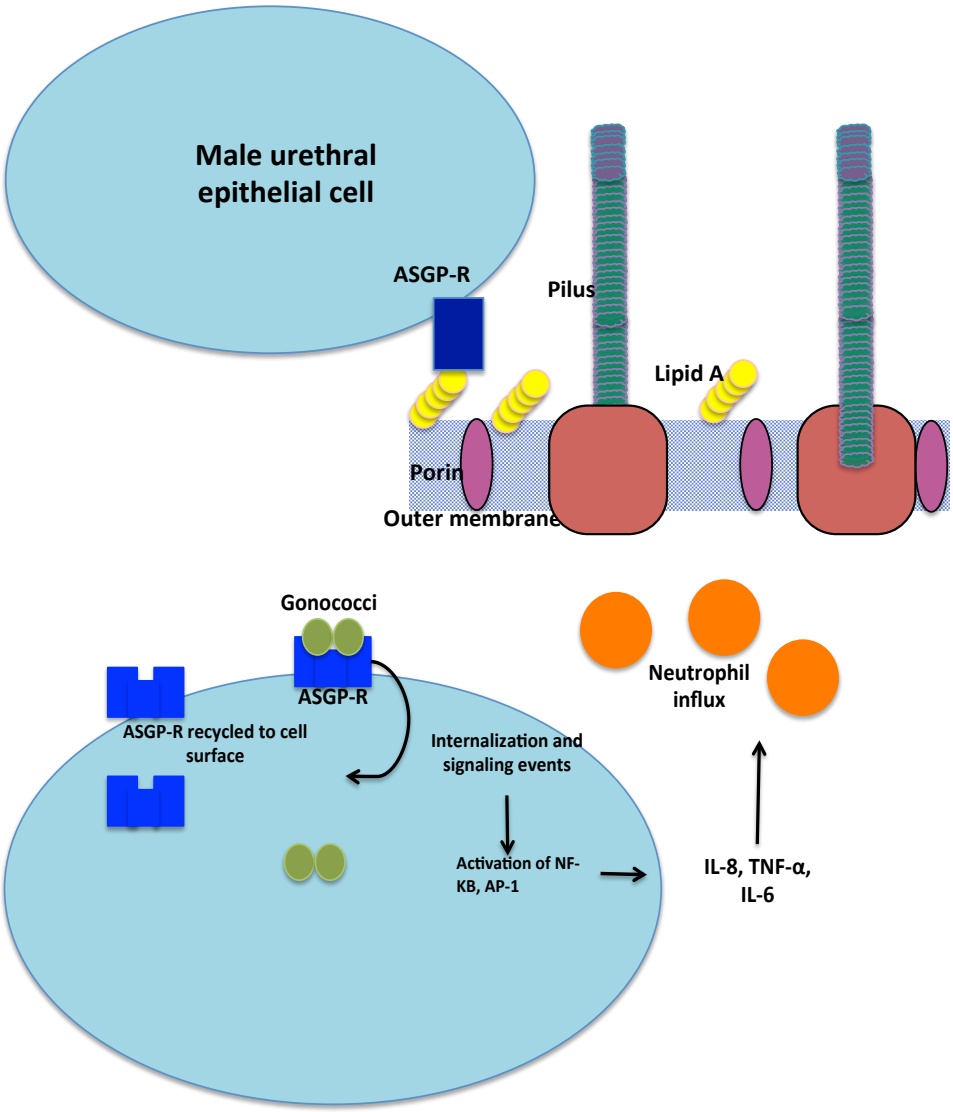
chemokines cause a large influx of neutrophils, resulting in the purulent discharge seen clinically. Porin is able to insert into the membrane of the phagocyte, inhibiting degranulation and the oxidative burst. This allows bacterial cells to survive within phagocytes. In women, gonococci are taken up by epithelial cells in the lower genital tract because of interactions with CR3 and CEACAMs. The bacteria trigger signaling events that promote uptake into epithelial cells and also produce a favorable environment within the host cell. Interestingly, this does not promote an inflammatory response, explaining the lack of symptoms in most females (Hedges, Mayo et al. 1999). *N. gonorrhoeae* also inhibits complement activity in women, contributing to the bacterium's ability to survive in the host (Edwards and Apicella 2004) The bacterium then is able to access sub-epithelial tissue and also proceed up the genital tract to cause the complications such as PID.

The gonococcus causes the most damage in the upper female genital tract. The lutropin receptors (LHr) that serve as receptors for gonococcal attachment in the endometrium and fallopian tubes are also found on the uterus, placenta, decidua, and fetal membranes (Reshef, Lei et al. 1990). Infection with gonorrhea increases the risk of spontaneous abortion, perhaps through attachment to these receptors. Gonococcal porin can translocate into eukaryotic cell membranes where it induces apoptosis. This is believed to be a factor in upper female genital tract infection, contributing to the cytotoxicity of the organism. Growing gonococcal cells release peptidoglycan monomers into the host environment. These monomers have been shown to be cytotoxic to fallopian tube cells (Melly, McGee et al. 1984; Cloud and Dillard 2002). Gonococcal LOS and pili are also toxic to the cells of the upper female genital tract (Johnson, Taylor-Robinson et

al. 1977; Gregg, Johnson et al. 1981; Gregg, Melly et al. 1981; Stephens, McGee et al. 1987; Velasquez, Garcia et al. 2012).

Figure 1.4 Infection of male urethral epithelial cells and the inflammatory response

The initial attachment between the epithelial cells of the male urethra and the gonococcus is through the pilus. Next, the gonococcal LOS binds to the asialoglycoprotein receptor on urethral epithelial cells, creating a tight association between the bacterium and the epithelium. The bacterial LOS elicits cytokine production by infected epithelial cells, causing an influx of PMNS and ultimately resulting in the symptoms seen in nearly all men with gonorrhea. (Figure adapted from (Edwards and Apicella 2004))



1.7.3 Serum Resistance

N. gonorrhoeae is able to evade normal human immune system responses. Antigenic and phase variation and the endless ability to modify surface structures allows the bacterium to easily escape recognition by the host. Gonococci are able to cause disseminated infection because the species has developed defenses against the complement cascade responsible for killing bacterial cells. Porin plays a large role in this serum resistance. PIA can bind Factor H, inhibiting the induction of the alternative complement pathway (Ram, McQuillen et al. 1998; Ram, Sharma et al. 1998). PIA can also bind to C4b-binding protein, inhibiting the classical complement pathway (Ram, Cullinane et al. 2001; Ram, Cullinane et al. 2001b). Sialylation of LOS allows the lipooligosaccharide to bind to Factor H or C4b (Ram, Sharma et al. 1998; Ram, Cox et al. 2003). These interactions cause a decrease in both the classical and alternative complement pathways.

1.7.4 Defenses against oxidative killing

N. gonorrhoeae encounters several sources of oxidative stress during infection of human hosts. Defenses against oxidative stress promote survival and thus increase the virulence of the organism. We propose that oxidative stress defenses might even play a role in antibiotic resistance (Chapter 4). Sources of oxidants are varied and include the by-products of the bacterium's own metabolic processes, oxidative products released after the host inflammatory response, and products of metabolic processes of commensal bacteria (Naids and Rest 1991; Zheng, Alcorn et al. 1994; Criss and Seifert 2008).

Oxidants have the potential to kill the gonococcus by damaging interactions with DNA, RNA, cellular membranes, and proteins (Imlay 2003).

The gonococcus has developed many mechanisms to defend itself against oxidative stress. The response to hydrogen peroxide (H_2O_2) has been studied extensively in the attempts to characterize oxidative defense pathways in *N. gonorrhoeae*. Microarray analysis showed changes in the levels of transcription of over 150 genes (Stohl, Criss et al. 2005). The OxyR regulon, consisting of *gor, prx,* and *katA,* is important for defenses against H_2O_2. Catalase converts harmful $H_2O_2$to oxygen and water. Catalase (*katA)* mutants are more sensitive to both $H_2O_2$and paraquat and the presence of *katA* increases resistance to *in vitro* killing and DNA damage from human PMNs and commensal bacteria (Zheng, Alcorn et al. 1994; Soler-Garcia and Jerse 2004).

A significant amount of superoxide ($O_2^{\bullet-}$) can be formed during aerobic respiration in *N. gonorrhoeae*. Under normal conditions, bacterial defenses are able to convert the $O_2^{\bullet-}$ into hydrogen peroxide, which is then managed by the peroxide defenses mentioned above. While superoxide dismutases (SOD) are important for detoxifying $O_2^{\bullet-}$ in other bacterial species, the manganese ion (Mn^{2+}) appears to play a major role in protecting against $O_2^{\bullet-}$ killing by quenching the toxic radicals (Tseng, Srikhanta et al. 2001). Though Mn^{2+} and manganese transporter are the major mechanisms for protecting *N. gonorrhoeae* from superoxide, low levels of *SodB* also contribute (Stohl, Criss et al. 2005).

N. gonorrhoeae is exposed to other reactive oxygen and nitrogen species (ROS and RNS) throughout the course of human infections. The pathogen has developed

complicated mechanisms involving many genes and pathways to avoid cellular damage and death caused by these toxic radicals.

1.8 Natural Transformation and Competence of *N. gonorrhoeae*

N. gonorrhoeae is remarkable in its ability to take up exogenous DNA from the environment and incorporate that DNA into its own chromosome, provided that it has ≥85% identity with DNA it replaces. This natural competence has allowed the gonococci to use transformation to acquire new genetic material. Proposed reasons for the uptake of DNA include the use of DNA as a nutritional source, aid in the repair of damaged chromosomes, acquisition of new virulence factors, and generation and spread of genetic diversity (Sparling 1966; Hobbs, Seiler et al. 1994). The gonococcus uses natural transformation as its only mechanism for mobilizing chromosomal loci and as its main mechanism of horizontal gene exchange (Sox, Mohammed et al. 1978; Koomey 1998). *N. gonorrhoeae* is one of approximately 70 bacterial species that are recognized as naturally competent (Johnsborg, Eldholm et al. 2007).

1.8.1 Natural competence of the gonococcus

Unlike most other naturally competent species, *N. gonorrhoeae* is nearly always competent; most other species regulate the expression of the proteins required for competence based on cellular needs or environmental signals. The competence genes of the gonococcus are constitutively expressed, a fact that is supported by the fairly easy ability to naturally transform the species in the laboratory. Gonococci have been reported to be highly competent during all phases of growth (Sparling 1966; Biswas, Sox et al. 1977). The presence of a ten base-pair uptake sequence (US) (5'-GCCGTCTGAA-3') is

required for the gonococci to take up DNA (Graves, Biswas et al. 1982; Goodman and Scocca 1988; Elkins, Thomas et al. 1991). This US is found approximately every 1000 base pairs in *N. gonorrhoeae* and is also found frequently in *N. meningiditis* and the commensal *Neisseria* species. Recent studies have shown that uptake of non-US containing DNA is possible and that the efficiency of this process is highly strain dependent (Duffin and Seifert 2010).

1.8.2 Sources of DNA for transformation

Since *N. gonorrhoeae* primarily takes up DNA with the *Neisseria*-specific US, the source of genetic material for transformation is most often a co-infecting *N. gonorrhoeae* strain in the human host; the exchange of chromosomal DNA during mixed infections is common (Hamilton and Dillard 2006). However, transformation is also possible between *N. gonorrhoeae* and other *Neisseria* species, such as the pathogenic species *N. meningiditis* or commensal species such as *N. cinerea*, *N. flavescens*, *N. sicca*, and *N. lactamica*, among others. In fact, transfer from commensal species was responsible for the creation of the allele that codes for a mutated penicillin binding protein that contributes to penicillin resistance in *N. gonorrhoeae* (Spratt, Zhang et al. 1989; Spratt, Bowler et al. 1992*)*.

Donor cells release DNA for transformation either through a type IV secretion system (T4SS) or through autolysis of the donor cell. Nearly 80% *N. gonorrhoeae* strains possess the gonococcal genetic island (GGI), which encodes a T4SS (Dillard and Seifert 2001). Genes of the GGI allow for the secretion of single stranded DNA into culture medium (Hamilton, Schwartz et al. 2001; Hamilton, Dominguez et al. 2005; Ito, Deguchi

et al. 2005). Gonococci can take up the DNA secreted by the T4SS and recombine it into their own chromosomes. This method allows for survival of the donor cell. Strains that do not carry a GGI must use autolysis as the source of genetic material for transformation. Autolysis occurs frequently in culture systems; cells are prone to autolysis during stationary phase or when exposed to non-ideal growth conditions (Morse and Bartenstein 1974; Hebeler and Young 1975; Elmros, Burman et al. 1976; Elmros, Sandstrom et al. 1976). *In vivo*, autolysis is probably mediated by amidases that break down peptidoglycan in the cell wall (Hebeler and Young 1976). Autolysis can also occur due to instability of the outer membrane (Cacciapuoti, Wegener et al. 1978; Bos, Tefsen et al. 2005). It has been shown that DNA released upon autolysis can be used for transformation (Norlander, Davies et al. 1979).

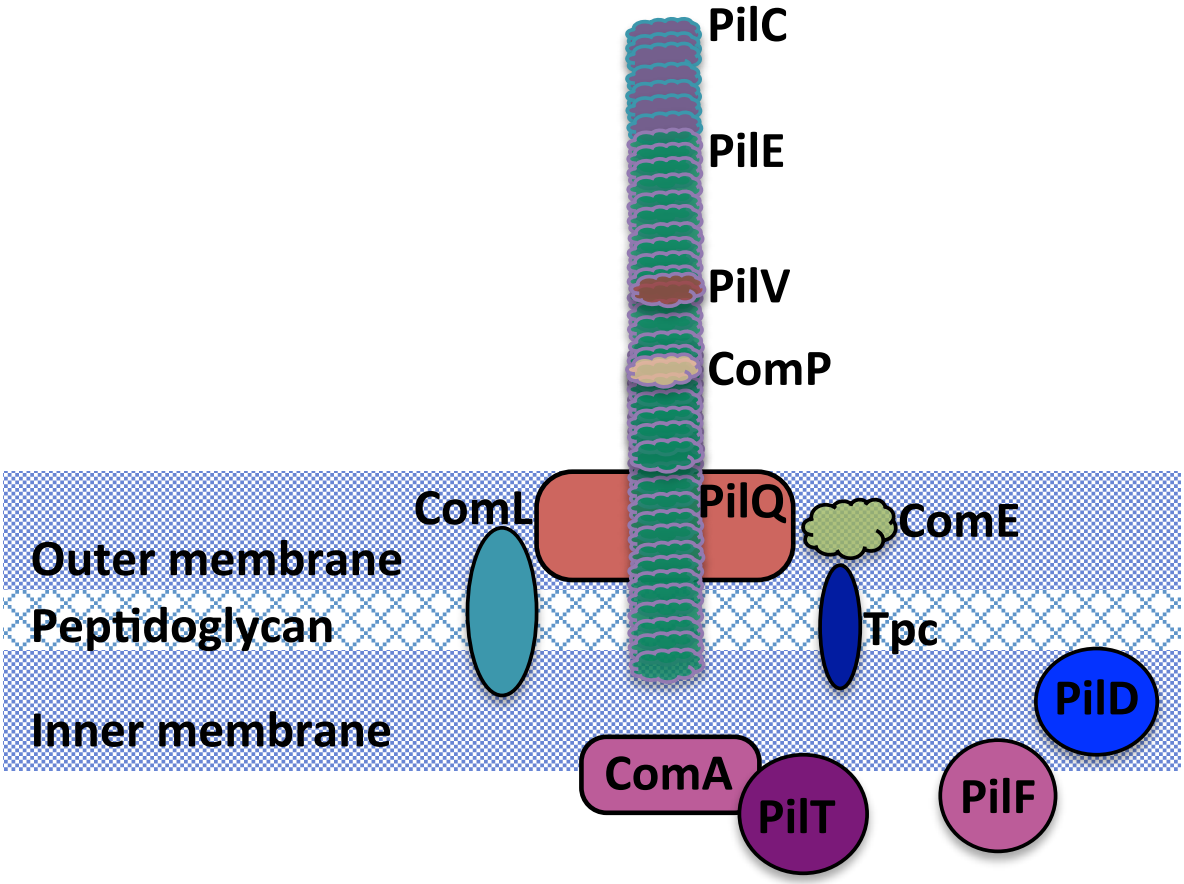
1.8.3 DNA binding and uptake

After DNA is released (either through a T4SS or through autolysis), the recipient cell must recognize and bind the DNA. DNA uptake and transport into the cell is separate from the initial binding of DNA to the cell surface. Binding and uptake are dependent on a variety of proteins. Piliated gonococci have a much higher transformation frequency than their non-piliated counterparts, indicating that there is a role in transformation for at least some of the genes involved in Tfp biogenesis and expression (Biswas, Sox et al. 1977). Opa proteins and ComP, a minor pilus protein, assist with dsDNA binding to the cell surface (Hill 2000; Aas, Lovold et al. 2002; Aas, Wolfgang et al. 2002). The Tfp is clearly involved in non-specific DNA binding, though a fully functional pilus is not necessary for DNA uptake (Boyle-Vavra and Seifert 1996; Zhao, Tobiason et al. 2005).

Mutations in *pilC, pilE, pilF,* and *pilG* have all been linked to a reduction in transformation efficiency (Rudel, Facius et al. 1995). PilQ, the pore through which the pilus is extruded, and other components of the secretin complex, such as PilM, PilN, PilO, and PilP, are required for transformation, since mutations in any of these proteins completely ablates transformation (Zhao, Tobiason et al. 2005). The pilus like protein ComP and PilV both affect binding of DUS containing DNA. ComE, found in the periplasm, binds DNA non-specifically and is believed to be important for DNA uptake once the DNA is has crossed the outer membrane (Chen and Gotschlich 2001). ComL, a lipoprotein, assists with creating an opening in the peptidoglycan layer for DNA entry (Fussenegger, Facius et al. 1996). ComA is found on the inner membrane and most likely assists with DNA transport from the periplasm to the cytoplasm (Facius and Meyer 1993). PilT, the NTPase necessary for retraction of the Tfp, is necessary for pulling the DNA, bound to the pilus or pilus-like apparatus, into the cell (Wolfgang, Lauer et al. 1998; Aas, Wolfgang et al. 2002).

Figure 1.5 DNA uptake and binding for transformation by *N. gonorrhoeae*

DNA binding and uptake requires the expression of several proteins. The pilus adhesion protein, PilC, and the major pilus protein, PilE, as well as the minor pilin protein PilV are involved. The secretin PilQ, the secretin complex proteins PilM, PilN, PilO, and PilP, and the pilus retraction protein PilT are also necessary. Pilus biogenesis proteins PilD, PilF, and PilG are necessary even though a fully functional pilus is not required for transformation. ComP, ComL, ComE, ComA, and Tpc also play roles in the DNA uptake process. (Figure adapted from (Hamilton and Dillard 2006).)



1.8.4 DNA processing

Gonococci can be transformed with either plasmid or chromosomal DNA, but transformation with chromosomal DNA is much more efficient (Eisenstein, Sox et al. 1977). Large plasmids are processed into linear dsDNA and are subject to restriction by endonucleases in the gonococcus (Stein, Gunn et al. 1995). Chromosomal DNA is not subject to the same restriction systems as plasmid DNA, perhaps because endonucleases do not act on ssDNA. Both dsDNA and ssDNA are subject to uptake by *N. gonorrhoeae*, but evidence shows that dsDNA is converted to ssDNA in the periplasm, which would be resistant to restriction modification systems (Biswas and Sparling 1981; Chaussee and Hill 1998).

1.8.5 Homologous recombination into the chromosome

Homologous recombination is the next step in natural transformation, but it is also involved with other processes in *N.* gonorrhoeae. In addition to using homologous recombination to incorporate exogenous DNA into its chromosome, the bacterium also uses homologous recombination to repair DNA damage and create antigenic diversity among pilins. The recombination pathways used for natural transformation, pilin angtigenic variation, and DNA repair are not fully understood and require different proteins (summarized in Table 1.2). Homologous recombination is dependent on RecA in *N. gonorrhoeae*, with RecA mutants showing a complete lack of transformation (Koomey and Falkow 1987; Koomey, Gotschlich et al. 1987). Other proteins involved in recombination include PriA, RecBCD, Rep, RecX, and RecN (Mehr and Seifert 1998; Stohl and Seifert 2001; Kline and Seifert 2005; Kline and Seifert 2005). In *E. coli,* the

RecBCD pathway is used to repair double stranded DNA breaks and the RecF pathway is used to repair single stranded breaks. Interestingly, RecX inhibits RecA mediated activities in *E. coli*, but enhances homologous recombination (mediated through RecA) in *N. gonorrhoeae,* indicating that though there are some similarities, the recombination pathways of these two bacteria are quite different (Gruenig, Stohl et al. 2010). While no RecF homologue has been identified in the gonococcus, genes encoding RecQ and RecO (both of which are involved in the *E. coli* RecF pathway) are present and are involved in homologous recombination in *N. gonorrhoeae,* but are not necessary for natural DNA transformation (Mehr and Seifert 1998). This RecF-like pathway in *N. gonorrhoeae* has instead been linked to homologous recombination events involved in pilin antigenic variation. RecJ has been characterized as an exonuclease involved in homologous recombination events necessary for pilin variation and DNA repair (Skaar, Lazio et al. 2002), while PriA is important for transformation events but is not necessary for pilin antigenic variation. RecN is also involved in recombination involved with transformation, but the exact mechanism is unknown. Rep, a helicase, is necessary for both transformation and antigenic variation, but is not linked to DNA repair events (Kline and Seifert 2005). Gonococci with a RecBCD-mutation have decreased transformation efficiency, but are still capable of intermediate-level transformation frequencies. Thus, a third recombination pathway, unrelated to RecBCD or the RecF-like pathway, is likely present in *N. gonorrhoeae*. It has been proposed that dsDNA utilizes the RecBCD pathway, while ssDNA utilizes a separate, as yet unidentified, mechanism. It is clear that the complex mechanisms involved in homologous recombination pathways in *N. gonorrhoeae* are not fully understood.

Table 1.2 Genes Involved in Homologous Recombination Processes in *N. gonorrhoeae*

N. gonorrhoeae uses homologous recombination for natural transformation, DNA repair, and pilin antigenic varation. While the pathways are not fully elucidated, some genes have been identified for each of the three processes. The "X" in the table below indicates that a gene is involved in the pathway.

	Natural Transformation	**DNA Repair**	**Pilin Antigenic Variation**
priA	**X**	**X**	
recA	**X**	**X**	**X**
recBCD	**X**	**X**	
recJ		**X**	**X**
recN	**X**	**X**	
recO		**X**	**X**
recQ		**X**	**X**
recX	**X**	**X**	**X**
rep	**X**		**X**

1.9 Antibiotic resistance in *N. gonorrhoeae*

The emergence of resistance to nearly all major classes of antimicrobials makes gonorrhea increasingly difficult to treat (Figure 1.7). In the 1930's, the sulfonamides were introduced and used to successfully treat infections with *N. gonorrhoeae*. However, by the mid 1940's, resistance to sulfonamides made the drugs useless against gonococcal infections. Sulfonamides were no longer recommended for the treatment of GC infections by 1949. Around the same time (the mid-1940's), penicillin was introduced as the drug of choice for gonorrhea infections. Early gonococcal isolates had a Minimum Inhibitory Concentration (MIC) of 0.004-0.01 μg/mL for penicillin and were thus extremely sensitive to the antimicrobial. In 1958, strains of *N. gonorrhoeae* with decreased susceptibility to both penicillin and streptomycin were reported (Cradock-Watson, Shooter et al. 1958). Over the next 40 years, the MICs of penicillin slowly increased, such that by the late 1970's and early 1980's the MICs of penicillin for some strains were greater than 2 μg/mL, indicating resistance to the antibiotic. By 1987, treatment failure with penicillin was widespread and the drug was no longer recommended for the treatment of GC infections. Also in the 1980's, increasing MICs of tetracycline became widespread, eventually leading to resistance and the withdrawl of tetracycline as a recommended treatment by option by the CDC. In the 1996, fluoroquinolones were introduced as a treatment for gonorrhea; however, resistance to this class of antibiotics began to develop soon after, and by 2007, the CDC stopped recommending the use of fluoroquinolones to treat GC infections because of the development of widespread resistance and treatment failure (Centers for Disease Control and Prevention 2007)(Centers for Disease Control and Prevention 2007)(Centers for Disease Control and

Prevention 2007)(Centers for Disease Control and Prevention 2007)(Centers for Disease Control and Prevention 2007)(Centers for Disease Control and Prevention 2007)(Centers for Disease Control and Prevention 2007). In the same year, the CDC added *N. gonorrhoeae* to its list of "superbugs", highlighting the difficulty in treating gonorrhea infections with resistance to beta-lactams, spectinomycin, tetracycline, azithromycin, and fluoroquinolones. The expanded-spectrum cephalosporins, cefixime and ceftriaxone, are currently the only antibiotics recommended against *N. gonorrhoeae*, but unfortunately resistance to these antibiotics is also increasing. As shown in Figure 1.7, the percentage of gonococcal strains with decreased susceptibility to ceftriaxone in Japan increased dramatically from 2001 to 2007. In 2009, reports of treatment failure with ceftriaxone and cefabuten began to emerge in the treatment of pharyngeal gonorrhea (Tapsall, Read et al. 2009). In 2010, a *N. gonorrhoeae* strain isolated in Japan was reported to have MICs of 2 μg/mL for ceftriaxone and 8 μg/mL with cefixime, and in 2011, a strain with MICs of 2 and 8 μg/mL for ceftriaxone and cefixime was isolated in France from a man who has sex with men (MSM) after he underwent treatment failure with cefixime (Ohnishi, Golparian et al. 2011; Unemo, Golparian et al. 2012). Both of these strains have MICs that are well beyond the levels considered as susceptible for these antibiotics (the resistance breakpoint is 0.25 μg/mL for both ceftriaxone and cefixime).

The Gonococcal Isolate Survelliance Project (GISP) reports data concerning antimicrobial susceptibility among gonococcal isolates collected in the United States each year. The most recent report found that 27.2% of isolates collected in 2010 were resistant to penicillin, tetracycline, ciprofloxacin, or a combination of those drugs. An increase in isolates with decreased susceptibility to azithromycin and cefixime, cefpodoxime, and

ceftriaxone was also observed. (Centers for Disease Control and Prevention 2011). While the United States has not yet reported the high-levels of resistance to the broad-spectrum beta-lactams that have been reported in Asia and Europe, susceptibility to these drugs is clearly decreasing and it is only a matter of time before they will no longer be useful for treatment of gonococcal infections.

Multiple labs, including ours, have explored the mechanisms by which *N. gonorrhoeae* becomes resistant to antimicrobials. Resistance to antibiotics can be either plasmid-mediated or chromosomally mediated. Plasmid-mediated resistance is observed for penicillin (expression of a β-lactamase) and tetracycline (production of TetM, a protective protein that prevents tetracycline action). For chromosomally mediated resistance, mechanisms include alterations of the drug target, modification of the drug, changes in permeability of the cell, and efflux of the drug out of the cell. *N. gonorrhoeae* has utilized all of these mechanisms. Table 1.3 summarizes some of the antimicrobial resistance mechanisms utilized by *N. gonorrhoeae*.

Table 1.3 Mechanisms of antibiotic resistance

Bacteria employ several different mechanisms to avoid killing by antibiotics. Some of these are intrinsic while others are acquired. The mechanisms can be broken down into four main categories shown in this table.

Mechanism	Description	Examples	Intrinsic or Acquired
Decrease in permeation across the outer membrane	Outer membrane gives intrinsic resistance by limiting entry to bacterium. Mutations altering permeability increase resistance significantly.	*penB*-Mutation in porB1b Increases resistance to penicillin, third generation cephalosporins and tetracycline. *penC*-mutation in *pilQ* reducing entry of penicillin and tetracycline (Zhao, Tobiason et al. 2005).	Both
Drug Modification	Bacteria can produce enzymes that alter antibiotics, resulting in decreased efficacy or complete uselessness of the antibiotic.	β-lactamases-Some strains of *N. gonorrhoeae* harbor plasmids that code for β-lactamases, which are enzymes that hydrolyze the β-lactam ring of penicillin and other β-lactam antibiotics (Ashford, Golash et al. 1976).	Acquired
Target Modification	Antibiotics work to kill bacteria through several different pathways. Bacteria have developed mechanisms to alter drug targets to escape killing by different antibiotics and these altered targets have spread throughout bacterial populations.	*penA*-Mutations in PBP2 that result in a decreased rate of acylation by penicillin were first described in 1974 (Sarubbi, Blackman et al. 1974). *rpsJ*-Mutation in the ribosomal protein S10 that results in high level tetracycline resistance (Hu, Nandi et al. 2005). *gyrA* and *parC*-Mutations in DNA gyrase and topoisomerase IV that cause resistance to fluoroquinolones (Belland, Morrison et al. 1994).	Acquired

Drug Efflux	Bacteria have several types of efflux pumps that function to remove antibiotics and waste products from the cell. These give a baseline level of resistance to the cell. Mutations have been identified that increase expression, thereby conferring increased resistance to various antibiotics.	MacAB-A promoter mutation has been identified that increases resistance to macrolides (Rouquette-Loughlin, Balthazar et al. 2005). MtrCDE-Mutations in the *mtr (multiple transferable resistance)* locus confer resistance to a wide range of antimicrobials (Maness and Sparling 1973). These mutations result in overexpression of the efflux pump.	Both

Figure 1.6 Timeline of antibiotics used in the treatment of *N. gonorrhoeae* infections

Since the introduction of the sulfonamides for the treatment of gonococcal infections in 1938, the bacterium has developed resistance to every class of drugs used to treat gonorrhea. Figure adapted from (Unemo and Shafer 2011).

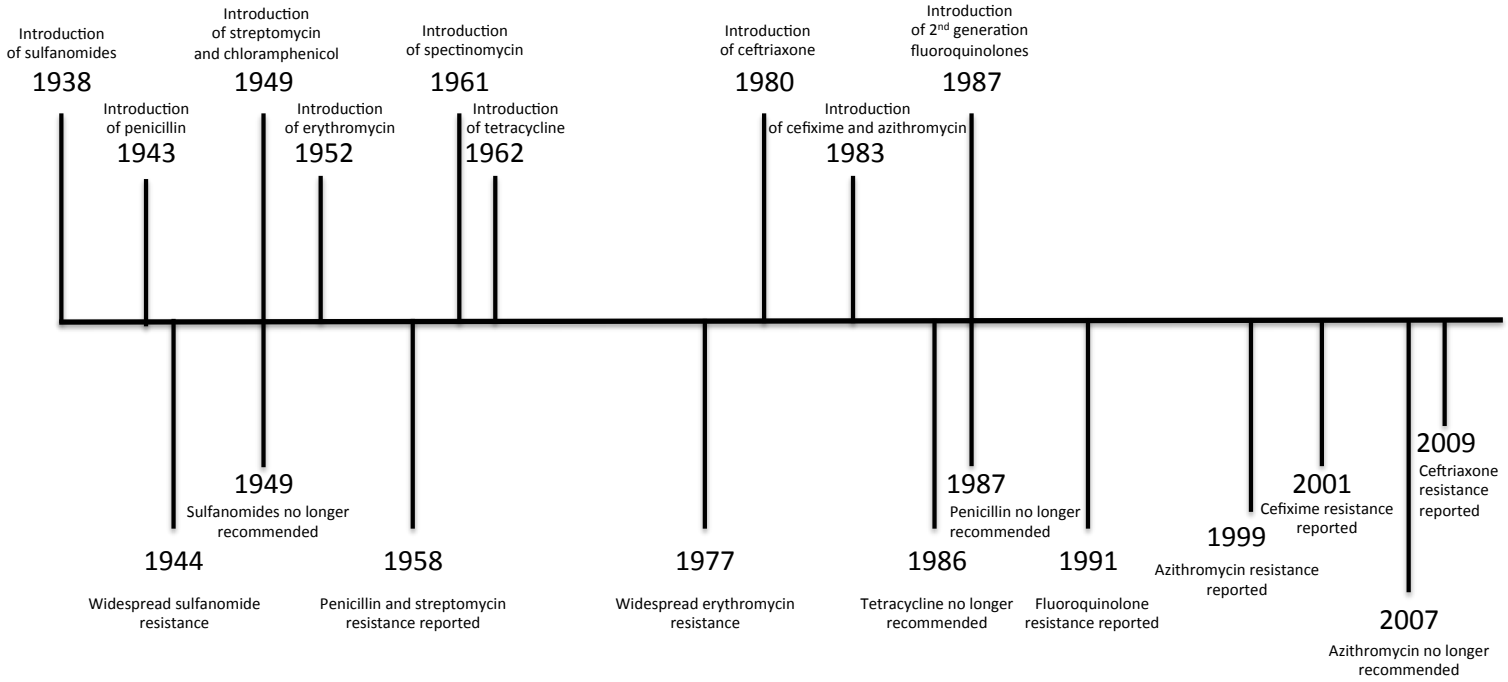
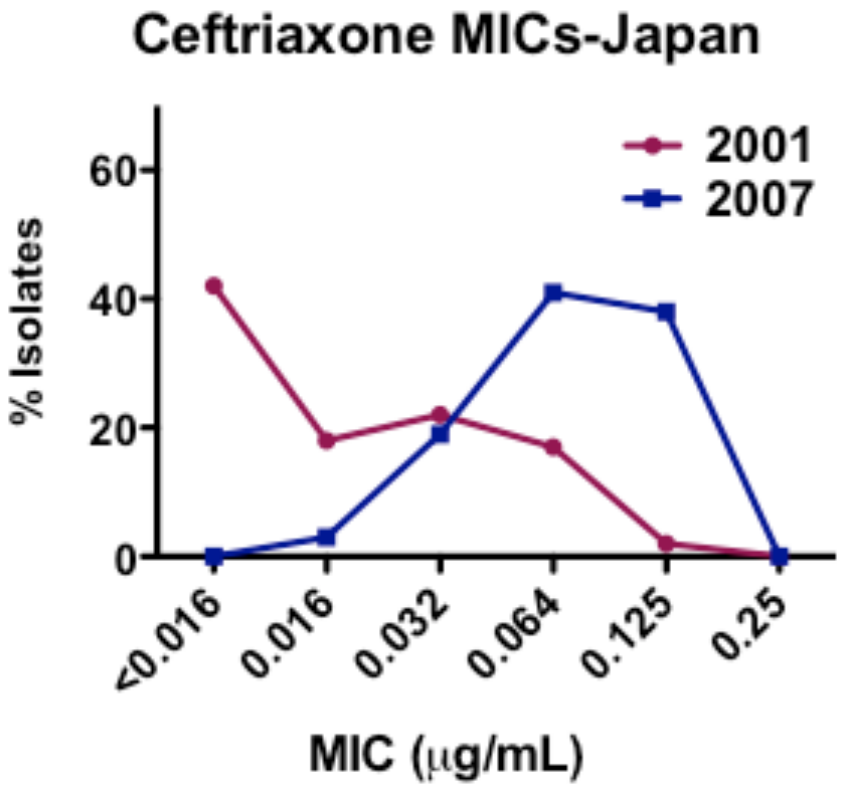


Figure 1.7 Increasing resistance to ceftriaxone in *N. gonorrhoeae*

The percentage of strains in Japan with decreased susceptibility to ceftriaxone increased dramatically from 2001 to 2007 (M. Ohnishi, personal communication).



1.9.1 Plasmid mediated resistance to penicillin and tetracycline

In 1976, two different types of beta-lactamase encoding plasmids were reported in Africa and Asia (Ashford, Golash et al. 1976; Phillips 1976). Since then, other plasmids encoding the β-lactamase enzyme TEM-1 have also been discovered (Dillon and Yeung 1989). Penicillin and other β-lactam antibiotics exert their antibacterial effects by interfering with cell wall synthesis in the bacteria. The antibiotic binds to the enzymes responsible for the cross-linking of the peptidoglycan layer that surrounds the cell and confers rigidity and viability to the bacterium. Disruption of a proper peptidoglycan layer results in bacterial cell death. Plasmid-mediated penicillin resistance involves the production of a TEM-1 β-lactamase, which hydrolyzes and inactivates the antibiotic. The gonococcal β-lactamase plasmid most likely was acquired from *Haemophilus ducreyi* (Anderson, Albritton et al. 1984).

Tetracycline binds to the 30S subunit of the bacterial ribosomes and inhibits protein synthesis by blocking the aminoacyl-tRNA from binding to the A site of the ribosome. Plasmid-mediated tetracycline resistance is caused by the TetM determinant, which encodes for a protein that protects to ribosome from tetracycline. The *tetM* gene is carried on a large conjugative plasmid that was originally found in bacteria from the *Streptococcus* genus and is also found in *Mycoplasma hominis, Ureaplasma urealyticum, and Gardnerella vaginalis*, which are pathogens that colonize the urogenital tract (Morse, Johnson et al. 1986). The plasmid is easily transferred between *N. gonorrhoeae* strains. The conjugative plasmid also mobilizes the β-lactamase plasmids, contributing to the spread of penicillin and tetracycline resistance. High-level plasmid-mediated tetracycline resistance was first identified in 1982 and within three years, strains harboring the *tetM*

plasmid were widespread throughout North American and Europe. Two variants of the tetracycline resistance plasmid have been identified, one is believed to have originated in the Far East and the other is most likely of African origin (Turner, Gough et al. 1999).

1.9.2 Chromosomally mediated resistance

Chromosomally mediated resistance involves the horizontal transfer of resistance determinants. Step-wise transfer of resistance genes occurs from a resistant strain to a sensitive strain through DNA uptake and homologous recombination (Cannon and Sparling 1984). Because *N. gonorrhoeae* is a naturally competent organism, chromosomally mediated resistance has been an efficient mechanism of spreading antibiotic resistance and is much more common than plasmid-mediated resistance. Approximately 12% of circulating gonococcal strains exhibit chromosomally mediated penicillin resistance, while only 1% of strains exhibit plasmid-mediated penicillin resistance (Centers for Disease Control and Prevention 2011). Chromosomally mediated resistance is more complex than plasmid-mediated resistance and involves the transfer of several genes.

1.9.2.1 Chromosomally mediated penicillin resistance

Chromosomally mediated resistance to penicillin involves the transfer of several genes in a specific order. Each of the genes individually increases resistance incrementally (2- to 5-fold), but when combined together, they result in a nearly 400-fold increase in the MIC of penicillin (Figure1.8). At least five genes are involved in chromosomally mediated penicillin resistance (Table 1.4). The first four determinants can

be transferred from penicillin-resistant strains to penicillin-sensitive strains in the laboratory, but transformation to high-level resistance equal to that of the donor strains has not yet been achieved.

The step-wise transfer of chromosomally mediated resistance begins with the transfer of a mutated *penA* gene, which encodes a mutated form of penicillin-binding protein 2 (PBP2), the essential PBP that catalyzes the transpeptidase reactions necessary cross-linking the peptidoglygan during cell wall synthesis. Mutations in *penA* likely were acquired by horizontal transfer from a commensal *Neisseria* species, *N. flavescens (Spratt, Zhang et al. 1989).* Altered PBP2 variants have a lower acylation rate with penicillin, resulting in a 4-6-fold increase in the MIC of the antibiotic. The most commonly mutated form of PBP2 from penicillin-resistant strains differs from wild type by an insertion of an aspartate at position 345a and 4-8 mutations in the C-terminal tail.

The second resistance determinant is a mutation in the *mtr* (multiple transferable resistance) locus. This mutation was described in 1973 as a mutation in the outer membrane that caused increased resistance to a wide variety of antimicrobials (Maness and Sparling 1973; Guymon and Sparling 1975; Guymon, Walstad et al. 1978). Later, it was discovered that the mutation actually caused an increase in drug efflux out of the bacterial cell because of increased activity of an efflux pump (Pan and Spratt 1994; Hagman, Pan et al. 1995). Overexpression of the MtrC-MtrD-MtrE efflux pump results from a single base pair deletion in the regulatory region of the gene (Hagman and Shafer 1995). This deletion increases transcription and relieves the repression caused by the MtrR repressor. The MtrR repressor binds and represses the transcription of the MtrC-MtrD-MtrE efflux pump (Lucas, Balthazar et al. 1997). *MtrC, mtrD,* and *mtrE* are

transcribed as a single transcriptional unit and are opposite from the regulatory *mtrR*, with the promoters of *mtrCDE* and *mtrR* overlapping. With the *mtrR* mutation, the spacing between the -10 and -35 regions of the MtrC-MtrD-MtrE promoter is disturbed, resulting in decreased binding of the repressor. While the most common mutation is this single base-pair deletion, other clinically relevant mutations that also result in the de-repression of the efflux pump also been described (Zarantonelli, Borthagaray et al. 2001; Cousin, Whittington et al. 2003; Cousin, Roberts et al. 2004). Overexpression of the efflux pump increases non-specific antimicrobial resistance by pumping antimicrobials out of the cell and by activating porin mutations (see below) that limit antimicrobial influx into the cell.

The third resistance determinant, termed *penB*, encodes a mutant form of the outer membrane protein porin, PorB1B ($porB_{1B}$). Alterations in loop 3, which folds into the barrel and constricts the pore, decrease diffusion of antibiotics across the outer membrane of *N. gonorrhoeae* (Gill, Simjee et al. 1998; Olesky, Hobbs et al. 2002*)*. Surprisingly, *penB* mutations are phenotypically silent in the absence of a co-resident *mtrR* mutation, suggesting a complex interaction between the two determinants (Olesky, Zhao et al. 2006). It is also important to note that *penB* mutations only appear in gonococcal strains expressing the PorB1B allele, but do not appear in strains with the PorB1A allele.

The fourth resistance determinant, *ponA*, encodes for an altered form of PBP1. The altered form has a lower rate of acylation for penicillin (Ropp, Hu et al. 2002). Surprisingly, the *ponA* determinant does not increase resistance when transformed into a 3rd level transformant (e.g. FA19 *penA mtrR penB*), but resistance decreases 2-fold when the *ponA* determinant is replaced with the wild-type *ponA* allele. A fifth resistance

determinant, which we call *Factor X*, is an unknown determinant that is required for high-level penicillin resistance. Unlike the other determinants, *Factor X* is not transformable in the laboratory, making its identification and characterization difficult.

Figure 1.8 Genetics of penicillin resistance

MICs of FA19 with known resistance determinants are shown. Each determinant only increases resistance incrementally, but when combined, there is almost a 400-fold increase in resistance between the susceptible strain (FA19) and the resistant strain (FA6140) (Maness and Sparling 1973; Sarubbi and Sparling 1974; Olesky, Hobbs et al. 2002).

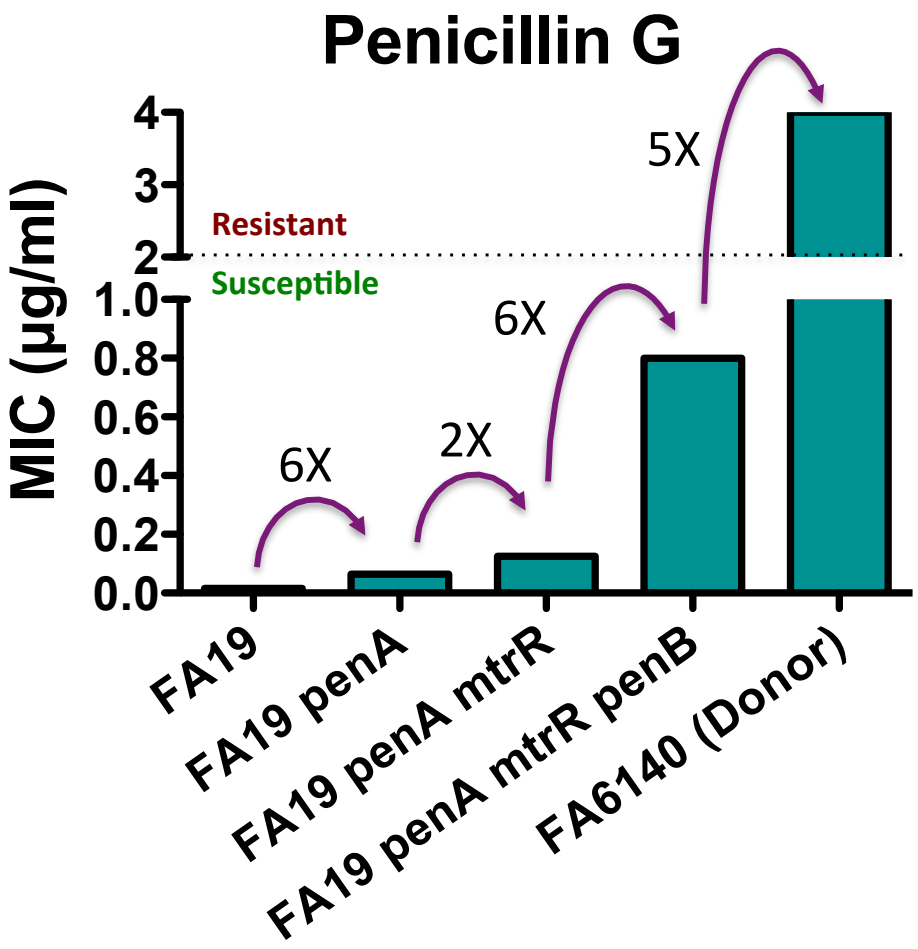


Table 1.4 Resistance determinants involved in chromosomally mediated penicillin resistance in *N. gonorrhoeae*

At least five genes are involved in high-level resistance to penicillin. The genes and their functions are listed.

Resistance Determinant	**Protein encoded /** Mechanism
penA	**PBP 2**/Altered forms that have a decreased acylation rate with β-lactam antibiotics
mtrR	**---**/Promoter mutation that increases transcription of the MtrC-MtrD-MtrE efflux pump that pumps hydrophobic agents and antibiotics out of the cell
penB	**$PorB_{1b}$**/Mutations in $PorB_{1b}$ porin at positions G120 and A121 in loop 3, which folds into the barrel and constricts the pore; requires the presence of the *mtrR* mutation for phenotype
ponA	**PBP 1**/Altered form that has a decreased affinity for β-lactam antibiotics
Factor X	**---/**Unknown determinant(s) that is(are) required for high-level (MIC ≥ 2 μg/ml) penicillin resistance

1.9.2.2 Chromosomally mediated tetracycline resistance

Chromosomally mediated resistance to tetracycline has been well characterized in *N. gonorrhoeae*. As with penicillin resistance, the acquisition of several genes is necessary for full resistance. In addition to their roles in penicillin resistance, mutations in *mtrR* and *penB* are also involved with resistance to tetracycline (Sparling, Sarubbi et al. 1975). A third determinant, *rpsJ1*, encodes for a mutation in the ribosomal binding protein S10 (Hu, Nandi et al. 2005). This mutation is necessary for high-level tetracycline resistance and likely disrupts the binding of tetracycline to the 30S subunit of the bacterial ribosome.

1.10 *N. gonorrhoeae* vaccine attempts

Because of the development of resistance to nearly all classes of antimicrobials, several groups have tried to develop a vaccine against *N. gonorrhoeae*. In fact, "vaccine therapy" was used even before antibiotics were available. Early vaccine attempts were simply whole cell lysates that were injected into joints (Stockman 1911). Surface structures are the obvious vaccine targets, but the ability of *N. gonorrhoeae* to quickly modify those structures makes vaccine design difficult. Additional complications exist because the gonococcus is able to suppress both CD4(+) T cell and B cell responses (Boulton and Gray-Owen 2002; Pantelic, Kim et al. 2005). Vaccines against the gonococcal pilus have been designed and did elicit an immune response, but are not effective because of the vast degree in variability among pilins (Schoolnik, Tai et al. 1983). No protection was provided from an experimental pilus vaccine given to members of the US military, likely because of antigenic variation among the pili (Anonymous

1983; Boslego, Tramont et al. 1991). Vaccines against the Opa proteins are also problematic due to variability in expression among, and within, strains (Hobbs, Seiler et al. 1994; Jerse, Cohen et al. 1994; Plummer, Chubb et al. 1994). While there is also much variability in LOS, studies show that conserved regions may be used as vaccine targets (Gulati, McQuillen et al. 1996; Gulati, McQuillen et al. 1996; Yamasaki, Koshino et al. 1999; Ngampasutadol, Rice et al. 2006). Studies utilizing porin as a vaccine target are underway since it is more antigentically stable than some of the other outer membrane structures (Wetzler, Blake et al. 1992; Zhu, Thomas et al. 2004; Wetzler 2010). A combination of a gonococcal antigen with a viral-like particle (VLP) or viral replicon particle (VRP) might provide the best approach to creating an effective vaccine against *N. gonorrhoeae* (Zhu, Chen et al. 2011).

Though vaccines for STIs remain controversial, the widespread use of the vaccine against human papilloma virus (HPV) might indicate a changing public opinion on the topic. Since being approved by the FDA in 2006, the two versions of the HPV vaccine have been given to approximately half of teenage girls (it was not initially approved for use in males). The topic of STI vaccines has already arisen with the development of this HPV vaccine. Thus when a vaccine for the prevention of gonorrhea is developed and approved, the public will hopefully be accepting and utilize it.

1.11 Introduction to the dissertation

Because of the important public health concerns associated with *N. gonorrhoeae* infections, it is important to further our understanding of the complex mechanism the pathogen uses to escape killing by antimicrobials. Emerging resistance to several

different classes of antibiotics portends a dismal future for the treatment of gonococcal infections. In order to design new therapeutics, we must first understand how resistance to current drugs developed. The goal of my work was to expand on our knowledge of the mechanisms of chromosomally mediated resistance, focusing primarily on β-lactam resistance.

1.11.1 Characterization of unique resistance determinants

A study of clinical isolates of *N. gonorrhoeae* collected in New Caledonia, located in the Pacific Rim, revealed a group of strains with intermediate levels of resistance to penicillin. These strains, however, harbored unusual mutations in *penB* and *mtrR* and were missing the more common mutations. I examined the phenotypes of these strains and determined the contribution of these mutations to resistance.

1.11.2 Characterization of genes found only in resistant strains of *N. gonorrhoeae*

I performed comparative analyses on four different strains of *N. gonorrhoeae*: 2 strains that are sensitive (FA19 and FA1090) and 2 that are resistant (FA6140 and 35/02) to penicillin. Interestingly, 55 unique genes/multi-gene clusters were present in both resistant strains but absent in the sensitive strains. I cloned each of these genes/multi-gene clusters into the gonococcal transfer/expression vector, pKH35, and transformed them into FA19 containing all of the known resistance determinants (FA19 *penA mtrR penB ponA)* and determined the penicillin MICs for each transformed strain.

1.11.3 Characterization of Factor X and high-level resistance

Because we were unable to transform sensitive strains to high-levels of resistance, we concluded that Factor X was likely not a single gene event. I wanted to try to learn more about the mechanisms underlying this high-level penicillin resistance. I initiated a set of experiments in which I reverted each of the known resistance determinants in a resistant strain (FA6140) back to wild-type and examined the phenotypes of the resulting revertant strains. I also performed bacterial killing assays, did transformation experiments, and determined the MICs of a wide variety of antibiotics with various strains to determine if Factor X is specific for penicillin resistance or whether it contributes to resistance of other antibiotics as well.

Chapter 2.

Characterization of Novel Resistance Determinants in *N. gonorrhoeae* Isolates from New Caledonia

2.1 Introduction

Gonococcal infections are a widespread problem, with the World Health Organization estimating there to be > 60 million cases worldwide. The public health problem of *N. gonorrhoeae* is complicated by the documented ability of the organism to rapidly evolve to become resistant to antibiotics and to generate antigenic diversity among strains. As a naturally competent organism, *N. gonorrhoeae* can take up DNA from the environment and incorporate it into its genome through homologous recombination. Commensal bacteria in the natural mucosal flora and other *Neisseria* species within the human host provide DNA for the bacterium to use to create diversity within its genome, particularly among the components of the outer membrane in order to escape recognition and killing by the immune system (Hobbs, Seiler et al. 1994). The natural ability of the organism to modify its genome has been a major contributor to the development and spread of antibiotic resistance genes (Cannon and Sparling 1984).

Penicillin was used to treat gonorrhea from the 1940's until the early 1980's. During this time, treatment with tetracycline was introduced because increased resistance to penicillin was routinely observed, but by the 1980s, widespread resistance to both antibiotics necessitated the move to other antibiotics. Since that time, the emergence of

resistance to nearly all major classes of antibiotics used to treat infections has followed, making *N. gonorrhoeae* an exeedingly difficult pathogen to treat. Penicillin resistance can arise via two different mechanisms: plasmid-mediated production of a beta-lactamase or chromosomally mediated transfer of resistance genes. Chromosomally mediated penicillin resistance, which is much more common than plasmid-mediated penicillin resistance, is also more complex and the mechanisms not fully elucidated. There are four known determinants (*penA, mtrR, penB, ponA*) and at least one unknown determinant that contribute to resistance. The four known determinants are transferred in a stepwise manner and individually increase resistance incrementally, but together cause a significant increase of ~400-fold. Both *penA* and *ponA* encode for altered forms of the two essential penicillin-binding proteins, causing a decrease in acylation and inhibition by β-lactam antibiotics (Dougherty, Koller et al. 1980; Ropp, Hu et al. 2002). Mutations in the regulatory region of the MtrC-MtrD-MtrE efflux pump (*mtrR*) result in overexpression of the pump, allowing the bacterium to more efficiently expel hydrophobic agents and antibiotics out of the cell (Pan and Spratt 1994). Finally, mutations in the major outer membrane porin, $PorB_{1B}$, decrease permeation of the antibiotic across the outer membrane into the periplasm (Guymon, Walstad et al. 1978; Gill, Simjee et al. 1998). The unknown determinant cannot be transferred by homologous recombination, but it is required for high-level penicillin resistance found in clinical isolates.

Similar to penicillin resistance, tetracycline resistance can be either plasmid-mediated or chromosomally mediated. Plasmid-mediated resistance is due to the acquisition and expression of the *tetM* determinant from members of the genus

Streptococcus (Morse, Johnson et al. 1986). Chromosomally mediated tetracycline resistance involves several genes, but unlike penicillin resistance, the mechanisms of resistance are better understood. Both *mtrR* and *penB* are required for intermediate-level resistance (Maness and Sparling 1973; Sparling, Sarubbi et al. 1975). A third determinant, *rpsJ1*, encodes a point mutation in the ribosomal binding protein S10, and is responsible for high-level tetracycline resistance (Hu, Nandi et al. 2005).

The major outer membrane porin of *N. gonorrhoeae* is either $PorB_{IA}$ or $PorB_{IB}$, which are encoded by one of two alleles found at a single locus ($porB_{1A}$ and $porB_{1B}$)(Knapp, Tam et al. 1984). Mutations in $porB_{1B}$ are important for resistance to both penicillin and tetracycline. Mutations in amino acids G120 and A121 of loop 3 of the protein are commonly observed and have been shown to reduce penicillin and tetracycline sensitivity (Olesky, Hobbs et al. 2002). Surprisingly, these *penB* mutations are phenotypically silent without a co-resident *mtrR* mutation (Olesky, Zhao et al. 2006; Shafer and Folster 2006). The MtrC-MtrD-MtrE efflux pump of *N. gonorrhoeae* is under transcriptional control of the MtrR repressor. The promoter for *mtrR* overlaps with the promoter for the *mtrCDE* operon (in the opposite direction). There is a 13-bp inverted repeat in the promoter region that serves as the binding site of the mtrR repressor (Lucas, Balthazar et al. 1997). While other mutations have been observed, by far the most common mutation in the *mtr* system is the deletion of a single nucleotide (-A) in the 13-bp inverted repeat (Warner, Shafer et al. 2008). The deletion both increases *mtrCDE* transcription and ablates *mtrR* transcription (Veal, Nicholas et al. 2002).

Public health officials in New Caledonia, located above Australia in the Pacific Rim, collected *N. gonorrhoeae* isolates over a three-year period to track the appearance

of known resistance alleles and monitor antibiotic resistance in the region. Historically, strains in this geographical area do not have the resistance alleles mentioned above and are still penicillin sensitive; thus penicillin G is still the most commonly used antibiotic for treatment of gonococcal infections. However, in this study, strains with intermediate level penicillin resistance were identified (MIC_{pen} = 0.133 mg/ml compared to MIC_{pen} = 0.017 mg/ml for susceptible strains). When the known resistance alleles were examined, these strains lacked the common mutations in both *mtrR* and *penB* that are usually associated with chromosomally mediated resistance, but they did have a common *penA* determinant (Vernel-Pauillac, Nandi et al. 2008). Instead of the commonly observed -A deletion in the *mtr* promoter, these penicillin intermediate-level resistant New Caledonia strains had a single base pair insertion (+T) in that same region (Figure 2.1). Moreover, these strains had mutations in $porB_{1B}$, but the common amino acid substitutions in loop 3 of porB were not present in the New Caledonia *penB*, and different substitutions were present both in loop 3 and elsewhere (Figure 2.2).

To determine the phenotypes of the novel mutations found in the *mtrR* and *penB* alleles isolated in New Caledonia, we compared strains with these *mtrR* and *penB* mutations to strains with the more common mutations in these genes. We show that the new *mtrR* mutation (+T in the inverted repeat of the promoter), in contrast to the –A mutation, does not contribute much to resistance to hydrophobic agents, and that the unusual *penB* allele found in the New Caledonia strains has a weaker phenotype than the more common *penB* alleles. Because New Caledonia is an isolated region, these new mutations appear to have arisen within the sexual network indigenous to this island

nation. As of present day, these mutations have not been found outside of the region and the more common mutations are not yet found in New Caledonia.

Figure 2.1 MtrR promoter sequences

The sequences of the MtrR promoter for FA19 (penicillin sensitive), FA6140 (penicillin resistant), and 319 (New Caledonia) are shown. FA6140 harbors the –A deletion typical of pen-resistant strains while 319 contains the +T mutation.

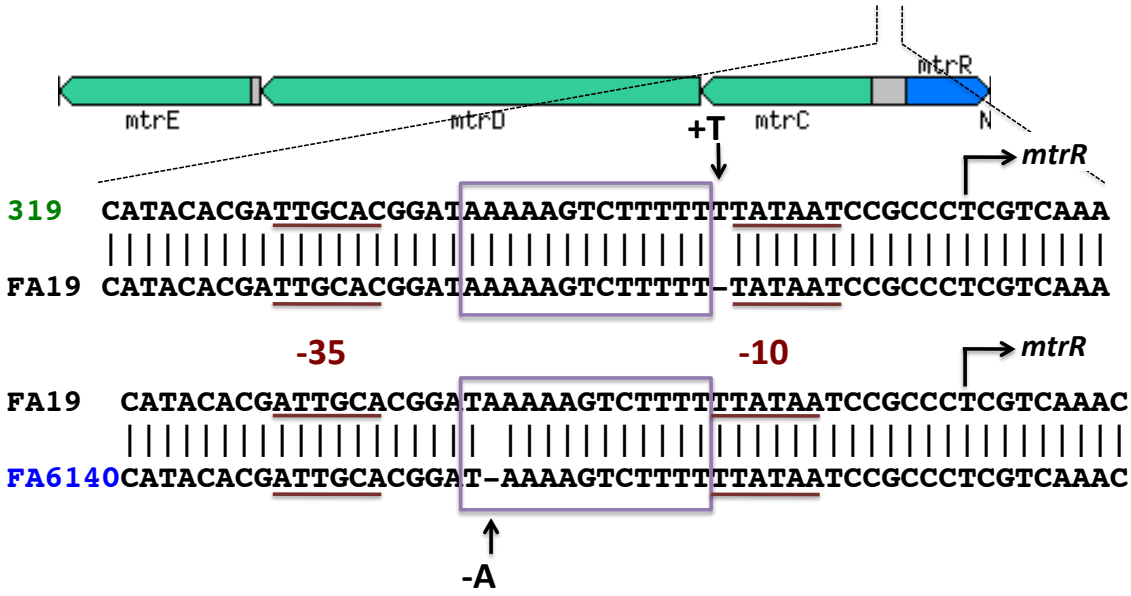
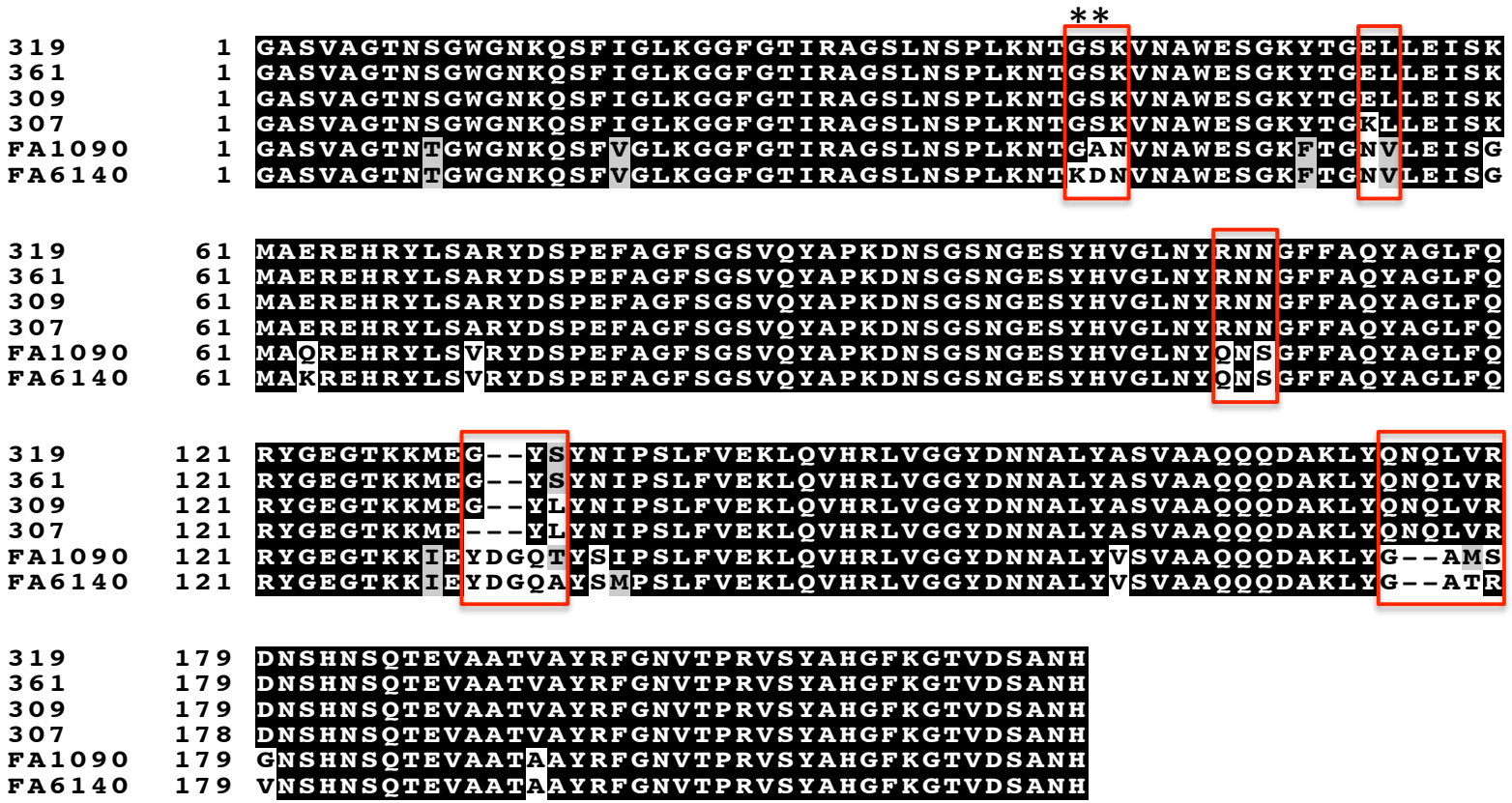


Figure 2.2 *penB* sequences from New Caledonia strains

Alignment of multiple *penB* alleles from New Caledonia and FA6140 and FA1090 (pen-sensitive). Red boxes highlight areas with significant sequence differences and * represents positions 120-121, where typical G120 and A121 *penB* mutations are found in penicillin-resistant strains.



2.2 Materials and Methods

2.2.1 Bacterial strains and DNA

N. gonorrhoeae strains FA19 and FA1090 (penicillin-susceptible laboratory strains) and FA6140 (a clinical isolate with a MIC pen=4 μg/ml) have been described previously and were kindly provided by Dr. Fred Sparling, University of North Carolina at Chapel Hill (Maness and Sparling 1973; Danielsson, Faruki et al. 1986). FA19Δ*mtrR* was provided by Dr. William Shafer, Emory University. Genomic DNA from the New Caledonia strains was provided by Dr. Cyrille Goarant, Institut Pasteur de Nouvelle-Calédonie. All strains used in this study are listed in Table 2.1.

2.2.2 Bacterial media and growth conditions

N. gonorrhoeae strains were grown on GC medium base (GCB) agar (Becton, Dickinson and Company; Sparks, MD) containing Kellogg's supplements I and II at 37°C with 5% CO_2 (Kellogg, Peacock et al. 1963). Liquid cultures of *N. gonorrhoeae* were grown in GC broth enriched with supplements I and II, supplement B, and 20 mM $NaHCO_3$ in a shaking incubator at 37°C (Hafiz and McEntegart 1976).

Table 2.1 *N. gonorrhoeae* strains used in this study

Strain	Description
FA1090	Pen and tet sensitive laboratory strain
FA19	Pen and tet sensitive laboratory strain
FA6140	CMRNG clinical isolate
FA19 *mtrR*	FA19 transformed with *mtrR* promoter mutation from FA6140
FA19 *mtrR*(319)	FA19 transformed with *mtrR* promoter mutation from 319
FA19 Δ*mtrR*	FA19 transformed with a plasmid containing a deletion in most of the coding region of *mtrR*. Described in Hagman 1995.
FA19 *penA mtrR*	FA19 transformed with *penA* and *mtrR* alleles from FA6140.
FA19 *penA mtrR* $porB_{1b}$	FA19*penAmtrR* transformed with plasmid containing $porB_{1b}$ from FA1090
FA19 *penA mtrR* $porB_{6140}$	FA19*penAmtrR* transformed with $porB_{1b}$ from FA6140 (*penB)*
FA19 *penA mtrR* $porB_{319}$#1	FA19*penAmtrR* transformed with $porB_{1b}$ from 319
FA19 *penA mtrR* $porB_{319}$#2	same as above
FA19 *penA mtrR* $porB_{(A102S)}$	FA19*penAmtrR* transformed with $porB_{(A102S)}$ mutant created by overlap PCR
FA19 *penA mtrR* $porB_{(N103K)}$	FA19*penAmtrR* transformed with $porB_{(N103K)}$ mutant created by overlap PCR

2.2.3 Preparation of DNA and PCR amplicands for genetic transformation

Gonococcal strains were grown on GCB agar and chromosomal DNA was isolated using the Wizard™ Genomic Purification Kit (Promega Corporation; Madison, WI) according to manufacturer's instruction.

The promoter region of *mtrR* and the coding sequences of $porB_{IB}$ genes were amplified by PCR with specific oligonucleotide primers using *Pfu* polymerase. FA1090 genomic DNA was used as a template in PCR reactions to create chimeras containing specific mutations. Chimeras containing the single por_{IB}-A102S and por_{IB}-N103K mutations and a construct containing both mutations together were constructed by overlap extension PCR (4-primer PCR, Figure 2.3). The $porB_{Ib}$ allele from the New Caledonia strain 319 was amplified for transformation into FA19 *penA mtrR* and FA1090 *penA mtrR*. Primers used for PCR are listed in Table 2.2. The products were analyzed by agarose gel electrophoresis and verified by sequencing. All PCR products were purified using the PureLink™ PCR Purification Kit (Invitrogen; Carlsbad, CA) according to manufacturer's instructions.

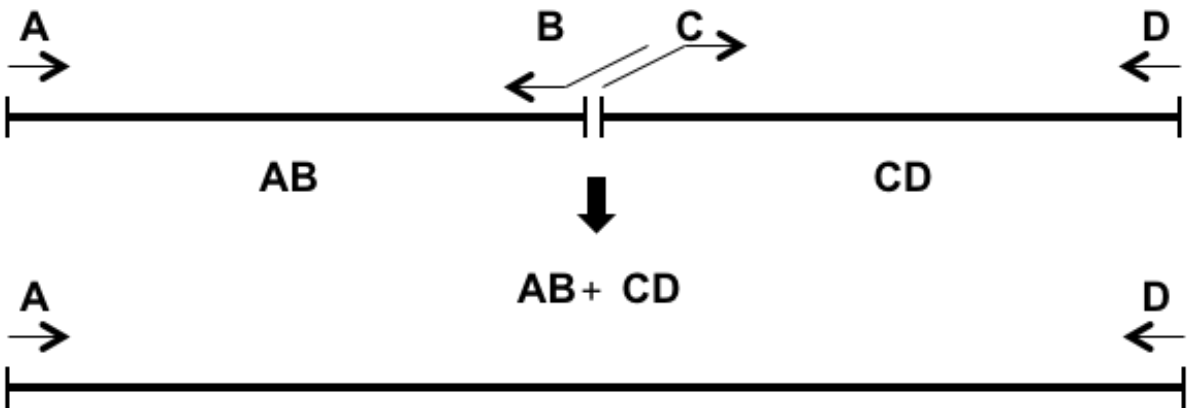
Table 2.2 Primers used for generation of *mtr* and *porB* mutants

Primers used for PCR preparation of amplicands for genetic transformation of *N. gonorrhoeae* are listed. Primers were synthesized by Eurofins MWG Operon (Huntsville, Alabama).

Primer	Sequence (5'->3' direction)
5'PorB-A121S	CCT GAA AAA CAC CGG CTC CAA CGT TAA CGC TTG GGA ATC CGG C
3'PorB-A121S	GCC GGA TTC CCA AGC GTT AAC GTT GGA GCC GGT GTT TTT CAG G
G'PorB-N122K	CCT GAA AAA CAC CGG CGC CAA AGT TAA CGC TTG GGA ATC CGG C
3'PorB-N122K	GCC GGA TTC CCA AGC GTT AAC TTT GGC GCC GGT GTT TTT CAG G
5'PorB-AN>SK	CCT GAA AAA CAC CGG CTC CAA AGT TAA CGC TTG GGA ATC CGG C
3'PorB-AN>SK	GCC GGA TTC CCA AGC GTT AAC TTT GGA GCC GGT GTT TTT CAG G
5'PorB-BsrGl/sil	GGC TTC AGC GGC AGT GTA CAA TAC GCA CC
3' PorB-BsrGl/sil	GGT GCG TAT TGT ACA CTG CCG CTG AAG CC
PorB-Hind-Fwd	GAG AAG CTT ACA AGA CTT CTC CAG CTT CC
PorB-BamH-Rev	AGA GGA TCC GGG TAA GTT TTA TCC ACG ACT
mtr-up	TTC AGA CGG CTG GAT GAT GCC GCC GAC TTG GC
mtr-down	GTC CAA GAA CCT CCT TCG GCA TCT

Figure 2.3 Overlap extension PCR to generate *porB*$_{1b}$ chimeras

AB and CD fragments were generated in the first round of PCR using the A,B and C,D primer pairs, respectively. The two fragments were united in the second round of PCR using the outside primers, A and D. This method was used to generate the chimeric *porB*$_{1bA102S}$, *porB*$_{1bN103K}$, and *porB*$_{1bAS>NK}$.



2.2.4 Genetic transformation

To generate *mtrR* and por_{IB} mutants in *N. gonorrhoeae*, genetic transformation of FA19 and FA19 *penA mtrR* was performed in GCB+ broth as described previously (Ropp, Hu et al. 2002). Briefly, cells from a -80°C frozen stock were streaked on GCB agar plates and incubated overnight at 37°C in 5% CO_2. The next day, a single piliated colony was passaged onto a fresh GCB plate and allowed to grow for 12-18 hours. Cells were swabbed from the plate and gently resuspended in prewarmed GCB+ broth and diluted to a cell density of 1 x 10^8 colony forming units (cfu)/ml (OD_{560} nm of 0.18). Between 2.5-5 μg of donor DNA (either chromosomal or PCR product) was diluted to 100 μl in 1X SSC and mixed with 900 μl of diluted cells in a round bottom polystyrene tube, with a loose lid to allow for aeration. Tubes were incubated for 5 h at 37°C in a humidified 5% CO_2 atmosphere. Aliquots (300 μl) of the transformation mixture were then spread onto GCB agar plates containing various concentrations of penicillin and grown overnight at 37°C in 5% CO_2. The next day, transformants were passaged onto fresh GCB plates. To verify positive transformants, individual colonies were resuspended in 30 μl of water, boiled for 5 minutes, and spun briefly to pellet the debris. Five microliters of the boilate supernatant was used as a PCR template and transformants showing correct recombination were confirmed by sequencing.

2.2.5 MIC determinations

Minimum inhibitory concentrations (MICs) of antibacterial agents were determined by the spot method on GCB agar containing antibiotics as previously described (Ropp, Hu et al. 2002). Briefly, non-piliated (P-) colonies were passaged for

two days on GCB agar plates, then resuspended in GCB+ broth and diluted to 1 x 10^7 cells/ml. Five microliters of diluted cells (50,000 cfu) were spotted onto GCB agar plates containing increasing concentrations of the appropriate antibiotic. The plates were incubated for 24 hours, and the MIC was defined as the minimal concentration of antibiotic at which no more than five colonies were observed following 24 hours of incubation.

2.2.6 RNA extraction

Gonococcal RNA was isolated and purified using a combined method of TRIzol (Invitrogen; Carlsbad, CA) and the RNeasy RNA extraction kit (Qiagen; Venlo, Netherlands). Liquid cultures of *N. gonorrhoeae* were grown in GCB broth as described above until they reached log phase (A_{560} = 0.4-0.5). Cells were harvested from 5 ml of log phase culture at 3000 x *g* for 5 min at 20ºC and resuspended in 3ml RNA later (Ambion; Austin, TX). Following incubation at 4ºC overnight, cells were pelleted and lysed with TRIzol (Invitrogen). Chloroform (1 ml) was added, and the phases were separated by centrifugation. The extracted RNA in the aqueous phase was further processed on the columns from the RNeasy Mini Kit according to manufacturer's instructions. The RNase inhibitor RNasin Plus (Promega; Madison, WI) was used to preserve RNA integrity and the RQ1 DNase kit (Promega) was used to remove residual DNA contamination. The quantity and quality of the RNA were checked by A_{260} and the A_{260}/ A_{280} ratio, as well as by visualization of the RNA on an agarose gel.

2.2.7 Real-time PCR

cDNA samples for qRT-PCR were prepared from 1 mg of RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Foster City, CA). Briefly, 10 µl of each RNA sample were mixed with random primers, dNTPs, RT buffer, and MultiScribe Reverse Transcriptase according to manufacturer's instructions. The mixture was incubated at 25°C for 10 minutes, followed by 120 minutes at 37°, and 5 seconds at 85°C.

Real-time PCR was performed on an ABI Prism 7500 Fast Real Time sequence detection system (Applied Biosystems; Foster City, CA). The cDNA was diluted and 100ng was combined with transcript specific primers and Absolute Blue™ QPCR Sybr green master mix (ABgene, Thermo Scientific; United Kingdom). Levels of the *mtrC* and *mtrR* transcripts were quantified using the comparitive C_T method, with *rnpB* (which encodes for ribonuclease P) used as the endogenous control. Primers for real-time PCR are listed in Table 2.3. All transcripts were assayed in triplicate on independent biological replicates.

Table 2.3 Primers used for real-time PCR

Primers used for amplification of *mtrC*, *mtrR*, and *rnpB* are listed. Primers were synthesized by Eurofins MWG Operon (Hunstville, AL).

Primer	Sequence (5'->3' direction)
mtrC 565F	GGC TTT ATC GGT CAG TCC AA
mtrC 641R	CGG ATG GTG GCT AAA ACA GT
mtrR-forRT	AAA ATT ACC GCC GTT TTG AC
mtrR-revRT	CCA ACG TCG ATT TGA TGA AG
rnpB-F	CGG GAC GGG CAG ACA GTC GC
rnp-R	GGA CAG GCG GTA AGC CGG GTT C

2.2.8 SDS Page and Western blotting

N. gonorrhoeae cells were grown overnight on GCB plates. The following day the cells were scraped from the plates and resuspended in GCB+ broth, diluted to an OD_{560} of 0.18, and 1 ml was removed and pelleted. The medium was removed, the pellet resuspended in 100 µl of SDS-PAGE loading buffer, and the samples were boiled for 5 minutes to lyse the cells. Samples (10 µl) were loaded onto 10% SDS-polyacrylamide gels and separated by electrophoresis. Proteins were transferred from the gel onto a Hybond-Low Fluorescing PVDF membrane (GE Healthcare; Piscataway, NJ) in a semi-dry transfer apparatus in 25 mM Tris, 102mM glycine, 10% methanol, 0.02% SDS. Following transfer, the membrane was blocked for 1h at 25°C in a phosphate-buffered saline with 0.1% Tween (PBS-T) and dry milk (5%). The membrane was then probed with a polyclonal MtrE-specific rabbit antibody (provided by Dr. Ann Jerse at Uniformed Services University of the Health Sciences) at 4°C overnight. PilW was used as a loading control. The blot was washed 3 times in PBS-T (PBS with 0.1% Triton X-100) and once in PBS, and the membrane was incubated for 2 h with an anti-rabbit Cy-3 labeled anti-rabbit secondary antibody (GE Healthcare). The blot was imaged on the Typhoon 9400 Imager (GE Healthcare) and ImageQuant software (GE Healthcare) was used for data analysis.

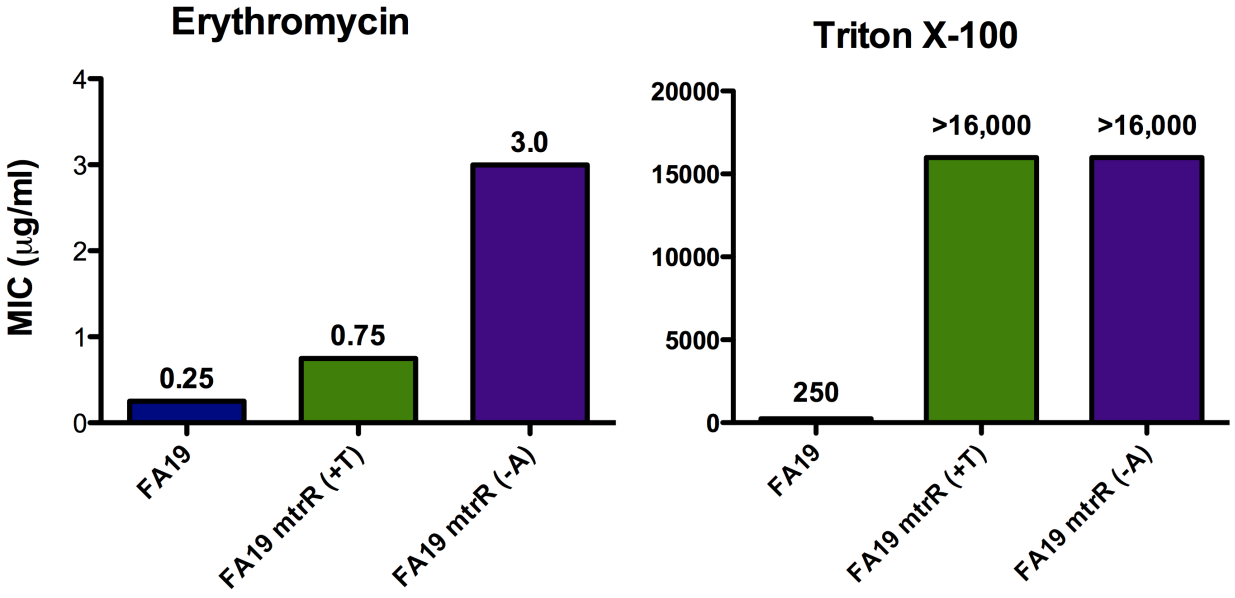
2.3 Results

2.3.1 The –A mutation confers higher levels of resistance than the +T mutation

N. gonorrhoeae strain FA19 was transformed with the PCR-amplified promoter region of *mtr* from the New Caledonia strain, 319, and levels of Triton X-100 just above the MIC (~250 μg/ml) were used for selection. Transformants were sequence-verified and their MICs were compared to MICs of FA19 containing the promoter from FA6140, a strain with the more common –A mutation. As shown in Figure 2.4, the +T insertion conferred high-level resistance to Triton X-100 (MIC > 16 mg/ml, the highest concentration that can be tested), but conferred only intermediate-level resistance to erythromycin (both Triton X-100 and erythromycin are subtrates of the MtrC-D-E efflux pump). In contrast, the –A deletion appears to have a stronger phenotype, conferring high-level resistance to both agents.

Figure 2.4 MICs of strains containing –A and +T mutations in the *mtr* promoter

MICs of FA19 transformed with the *mtr* promoter regions of FA6140 (-A mutation) and a strain from New Caledonia, 319, (+T mutation) are shown. Both mutations give full resistance to Triton X-100 but the –A mutation confers higher resistance to erythromycin than the +T mutation does.



2.3.2 Effect of the +T mutation on transcription and translation of Mtr genes

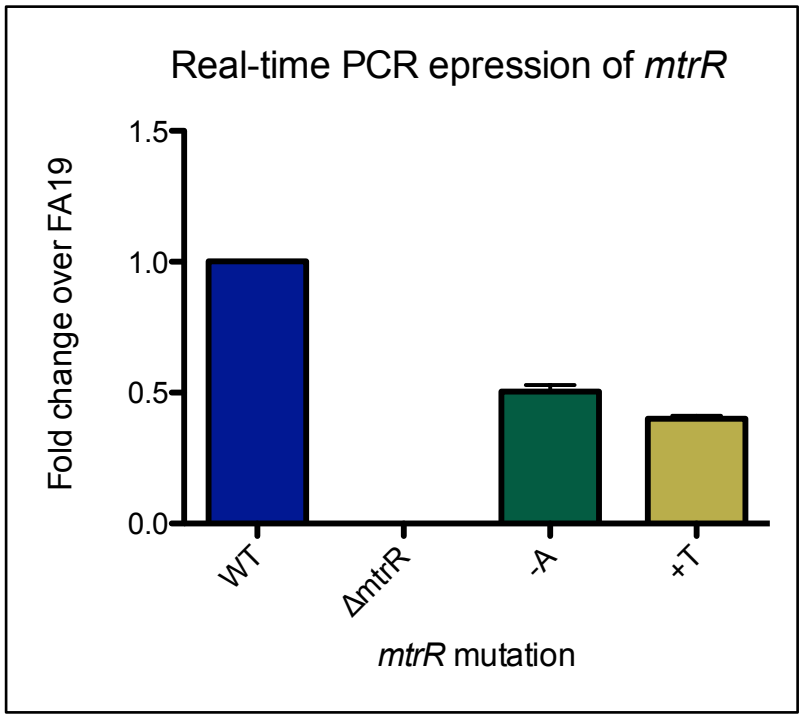
The +T mutation could have several distinct phenotypes: a) it could interfere with *mtrR* transcription, thus decreasing the amount of MtrR available for inhibition of *mtrCDE* transcription; b) it might block binding of MtrR to its operator, thus releasing its inhibitory block on *mtrCDE* transcription; or c) a combination of both. To investigate the effects of the +T mutation, we used quantitative real-time PCR to quantify RNA levels of the *mtrR* transcriptional repressor and of *mtrC*, the first gene of the mtrCDE efflux pump operon that is regulated by *mtrR*. We extracted RNA from four strains of *N. gonorrhoeae* with different *mtr* mutations for analysis.

Both the –A and +T mutations significantly decreased transcript levels of *mtrR*, with the –A deletion resulting in the largest decrease. As expected, the strain containing an *mtrR* deletion showed no *mtrR* RNA levels. Moreover, these experiments showed that the –A deletion resulted in a 6-fold increase in transcription of *mtrC* over that in FA19, whereas the +T insertion resulted in only a 1.5-fold increase, similar to the increase seen in a Δ*mtrR* strain (Figure 2.5).

We also used quantitative Western blotting to compare the effects of the +T and –A mutations on protein expression of MtrE. Using whole cell lysates from the same four strains used in the RT-PCR studies, we probed blots with an antibody to MtrE. The levels of protein were consistent with our quantitative real-time rtPCR results: the -A deletion resulted in the largest increase in MtrE (12- fold over wild-type), while the +T insertion had a more modest effect, a 2-fold increase over wild-type, similar to the increase seen in the *mtrR* deletion strain (Figure 2.6).

Figure 2.5 Transcription of *mtrR* and *mtrC* in strains with –A, +T, and Δ*mtr* mutations.

Results from quantitative real-time PCR are shown. Figure on left shows that *mtrR* transcription was absent in the *mtrR* deletion strain and decreased by about half in the strains with single amino acid deletions in the promoter. Transcription of *mtrC* was increased the most with a –A deletion.



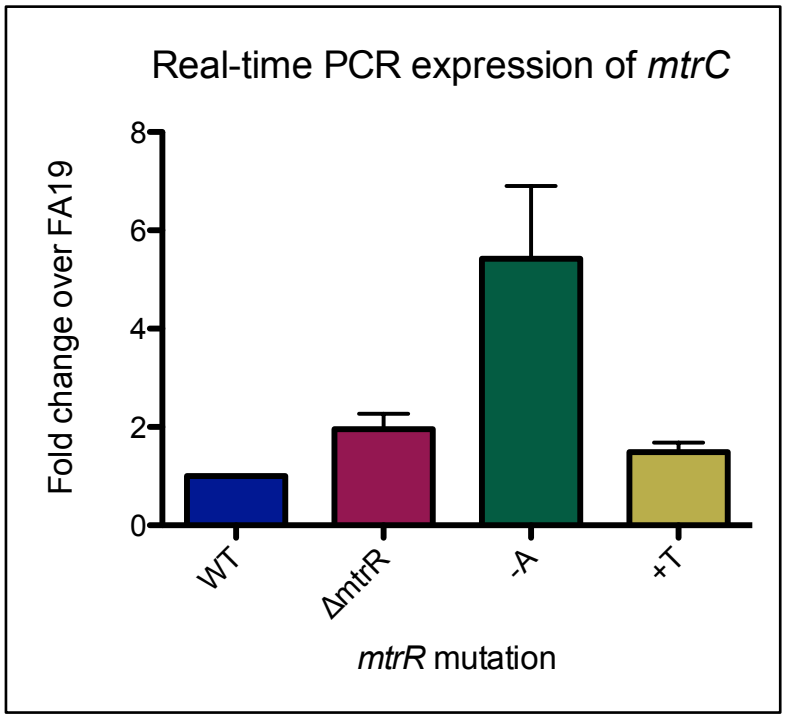
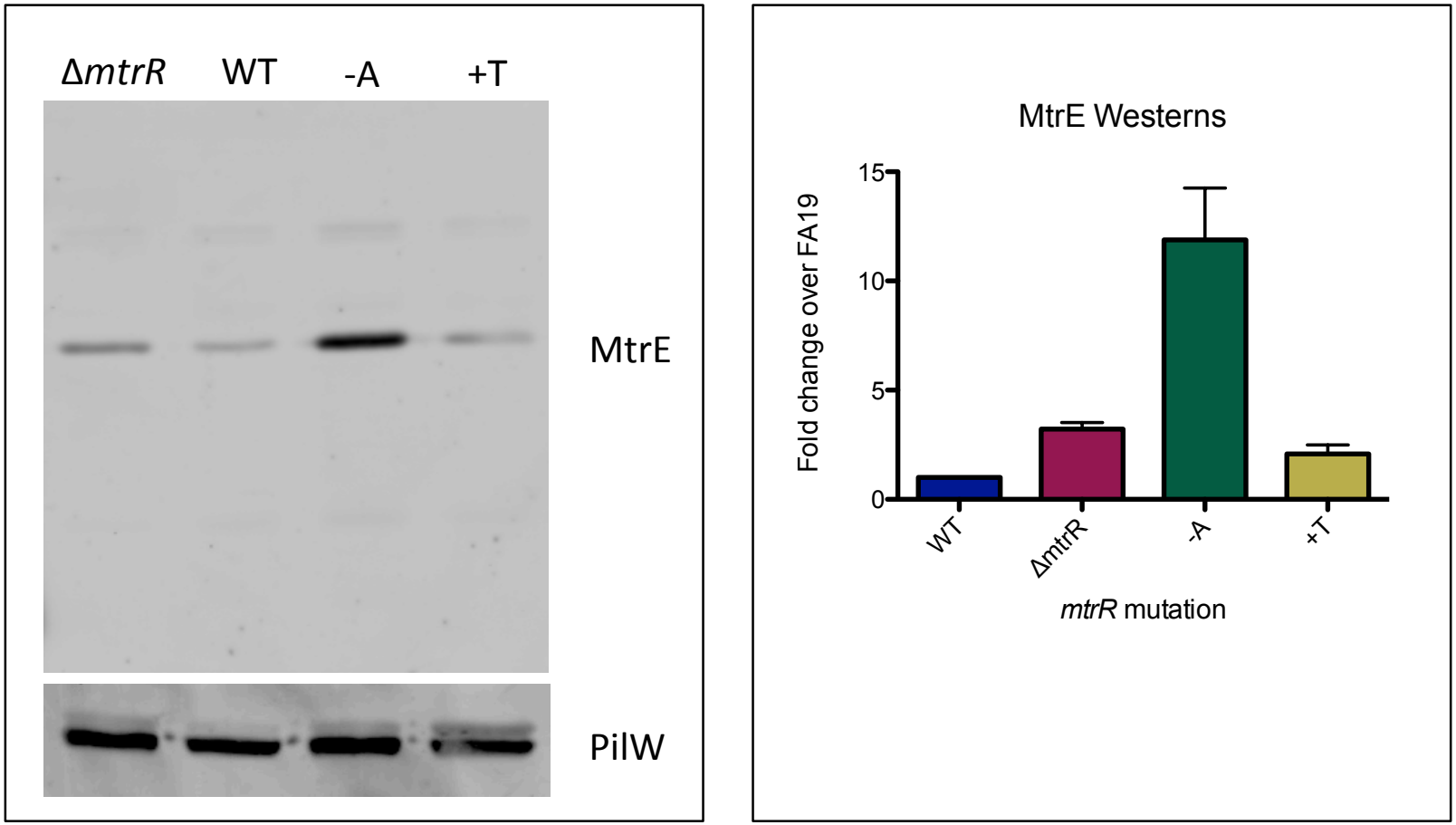


Figure 2.6 Effect of +T mutation on expression of MtrC
Quantitative Western blotting was performed to compare the effects of the -A and +T mutations on expression of the Mtr efflux pump. The -A increases expression more strongly than the +T does.



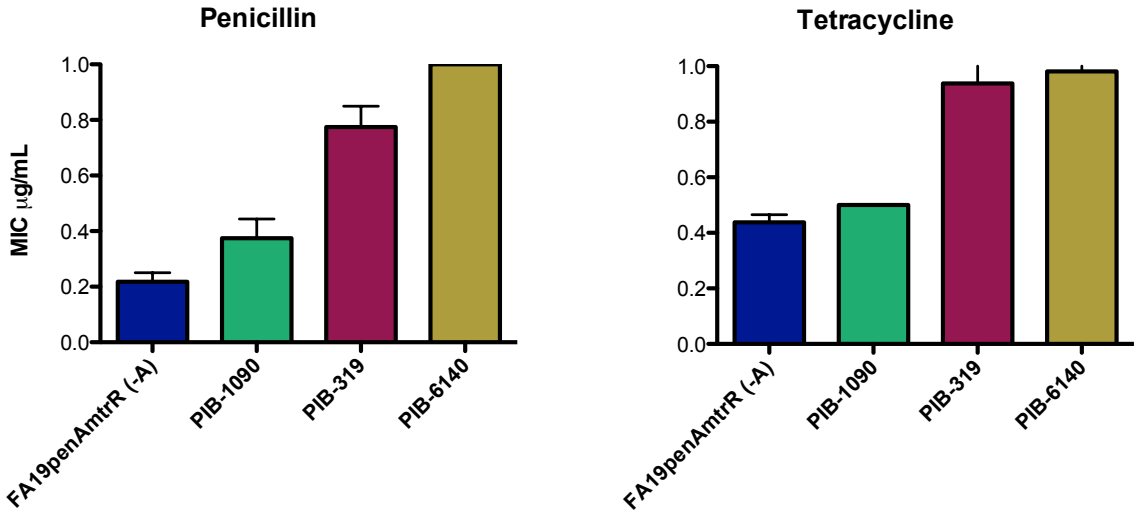
2.2.4 The effect of 319 *penB* on penicillin and tetracycline resistance

Since unique mutations in $porB_{1b}$ have been identified in strains from New Caledonia, we explored the contributions of these mutations to resistance. We first transformed the $porB_{1b}$ allele from strain 319 into FA19 *penA mtrR*. This recipient strain harbored the *penA4* gene from the penicillin-resistant strain FA6140 along with the common –A deletion in the *mtrR* promoter region, because we have shown previously that the *penB* determinant requires the presence of the *mtrR* determinant to confer resistance. The MICs of penicillin and tetracycline were determined as shown in Figure 2.7. The strain harboring the 319 $porB_{1b}$ allele exhibited intermediate resistance to penicillin but equivalent levels of resistance to tetracycline as the strain with the $porB_{1b}$ allele from FA6140.

To determine which specific residues in the *penB* allele from the New Caledonia strain 319 are important for resistance, we first focused on the mutations found in loop 3 of the New Caledonia porin. These mutations (A121S, N122K) were incorporated both individually and together into the wild-type $porB_{1B}$ allele from FA1090; the mutated $porB_{1b}$ alleles then transformed into FA19 *penA mtrR*. The MICs of penicillin and tetracycline were determined. As shown in Figure 2.8 the N122K mutation had no effect on resistance to penicillin or tetracycline, while the single A121S mutation conferred the same level of penicillin resistance as the *penB* allele from 319. In contrast, the A121S mutation conferred only a portion of the full resistance to tetracycline, suggesting that resistance to tetracycline requires additional mutations.

Figure 2.7 MICs of FA6140 and 319 *porB*$_{1b}$ alleles to penicillin and tetracycline

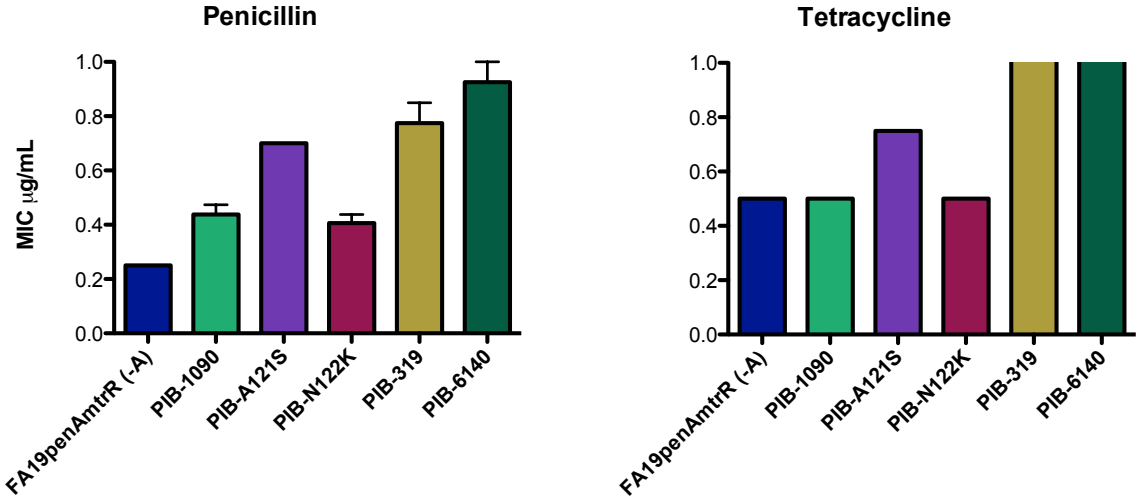
Transformation of *porB*$_{1b}$ alleles from FA6140 and 319 increased resistance to tetracycline equally. FA6140 *penB* increases resistance to penicillin more than 319 *penB* does.



Parental strain: FA19 *penA mtrR (-A)*

Figure 2.8 Identification of mutations in *penB* important for resistance

MICs of penicillin and tetracycline. Parental strain was FA19 *penA mtrR*. The A121S mutation is responsible for most of the resistance to penicillin, but additional mutations appear to be necessary for resistance to tetracycline.



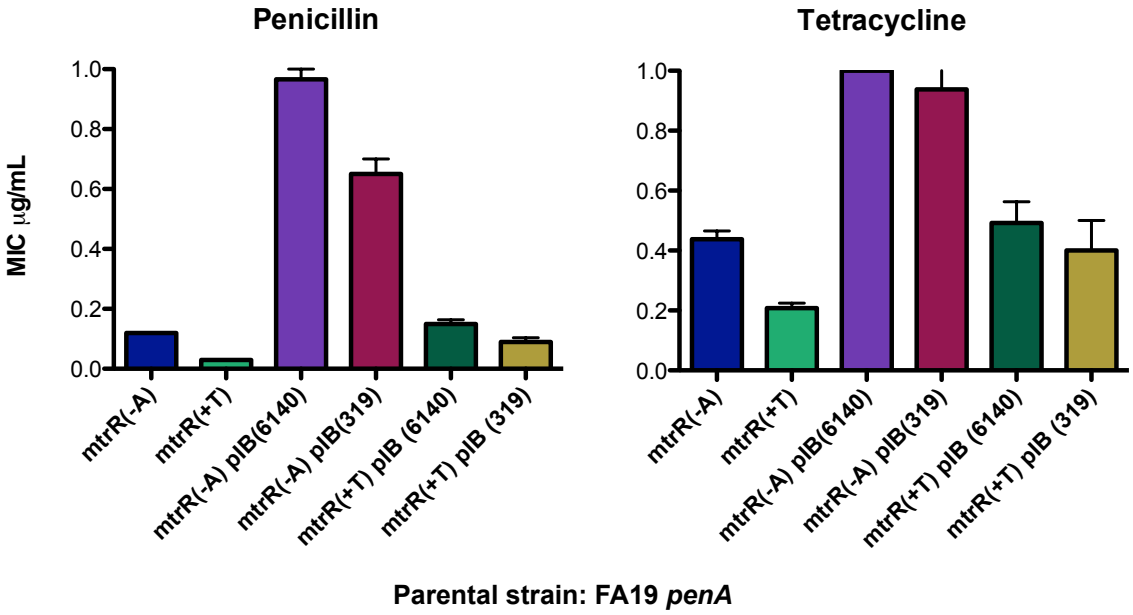
Parental strain: FA19 *penA mtrR (-A)*

2.2.6 The combined effects of the New Caledonia mutations

We also created strains of FA19 *penA* containing the unique *mtrR* mutation (+T insertion) from strain 319 and the *penB* alleles from both FA6140 and 319. Surprisingly, there was no significant increase in resistance in any strain constructed with the +T insertion mutation, indicating that this allele is not capable of activating either *porB* allele (Figure 2.9). These data are consistent with the results above showing that the +T mutation only weakly increases *mtrCDE* transcription in comparison to the –A deletion.

When we attempted to transform FA19 *penA mtrR penB* with genomic DNA from strain 319, no transformants were obtained. Since none of the known determinants from 319 (*penA, mtrR, penB*) fully explained the increased resistance seen in this strain, another factor must play a role in penicillin resistance. We attempted to transform this unidentified determinant into FA19 but had no success, indicating that the cause of increased resistance is not transformable. This is consistent with our studies in other resistant strains, including FA6140 and 35/02.

Figure 2.9 Both the *penB* and the *mtr* mutations found in New Caledonia strains have weaker phenotypes than more common mutations



2.4 Discussion

Because mutations in both *mtr* and $porB_{1b}$ are known to contribute to antibiotic resistance, these alleles are often sequenced among clinical isolates of *N. gonorrhoeae*. I characterized the unique mutations that had been found in the clinical isolates from New Caledonia with intermediate-levels of penicillin resistance. Our studies showed that the +T mutation in *mtrR* from strain 319 produces a much weaker phenotype than the more commonly found -A mutation does. We were able to show that this mutation increases resistance to erythromycin, penicillin, and tetracycline, but the increase is relatively small. Real-time quantitative PCR showed that the *mtrR* (+T) mutation increases transcription of *mtrC* by approximately 1.5 fold, while the -A mutation increases transcription by 6-fold. Quantitation of Western blot revealed a 2-fold increase in expression of MtrE with the +T mutation and a 12 fold increase with the -A mutation. The other resistance determinant from strain 319 with uncommon mutations, $porB_{1B}$, increases resistance to penicillin but not to the levels seen with the *porB1b* allele from FA6140. The A121S mutation appears to be responsible for most of the resistance to penicillin but other *porB* mutations are likely required to achieve full resistance in tetracycline. The N122K mutation did not seem to play a role in resistance to either antibiotic.

The New Caledonia strains examined here were a subset of large cohort of strains investigated for their resistance profiles and the sequence of their common resistance determinants. While none of the strains examined had high-level penicillin resistance, the subset of strains including strain #319 had much higher MICs of penicillin (0.136 μg/ml vs 0.017 μg/ml for the sensitive strains) than the others, and surprisingly they contained

unusual mutations in both their *mtrR* and *penB* determinants. We set out to examine whether these unusual alleles were responsible for the higher than expected MICs, but in fact these determinants had much weaker phenotypes than the determinants from FA6140, a penicillin-resistant strain that we have characterized in much detail. When we transformed FA19 *penA mtrR penB* (irrespective of whether the determinants were from FA6140 or 319) with chromosomal DNA from 319 in anticipation that these strains contained an additional transformable determinant, no transformants with lower penicillin susceptibility could be selected even at the lowest selection concentration of penicillin. These data suggest that 319 and similar strains were likely clonal variants with higher intrinsic resistance to penicillin than "normal" wild-type gonococci. One intriguing possibility is that these strains contained the elusive Factor X, the non-transformable, potentially polygenetic determinant that is responsible for high-level resistance in FA6140.

The importance of the *mtr* system to antibiotic resistance was first recognized in 1974 (Maness and Sparling 1973). Proteins of the efflux pump were characterized over the next 20 years, but it was not until 1994 that the repressor MtrR was first described and the regulation of the *mtr* efflux pump by *mtrR* was implicated in antibiotic resistance (Pan and Spratt 1994). Several mutations in *mtrR* have been identified that increase resistance to various antibiotics. Other mutations in *mtrR*, including an insertion of a TT in the promoter or missense mutations in the coding region are associated with intermediate and low levels of antibiotic resistance, respectively (Hagman, Pan et al. 1995; Hagman and Shafer 1995; Shafer, Balthazar et al. 1995; Warner, Shafer et al. 2008). Before studies of these New Caledonia isolates performed by our lab and our

collaborators, the +T mutation was not linked to antibiotic resistance (Cousin, Whittington et al. 2003; Cousin, Roberts et al. 2004). However, our results clearly disagree with this conclusion and the +T mutation does increase resistance to antibiotics through modulation of the MtrC-D-E efflux pump expression. We have shown that the -A mutation has a stronger phenotype than +T *mtrR* promoter mutations found in the New Caledonia strains, contributing to higher levels of resistance to antimicrobials.

It is interesting that even the weaker +T insertion in the promoter region of *mtr*, which increases MtrC-MtrD-MtrE expression by ~2-fold, gives full resistance to Triton X-100 without conferring full resistance to erythromycin or other antibiotics. While the implications of this result are unclear, apparently only a small change in *mtrCDE* transcription is capable of providing very high levels of resistance to detergents. Another possibility is that changes in *mtrR* transcription have effects on other proteins involved in Triton X-100 resistance. One such protein could be MtrF, which previously has been implicated in detergent resistance (Veal and Shafer 2003).

Previous studies have shown that the $porB_{1b}$ allele is involved in antibiotic resistance. A mutation at position 120 (G→K) in FA6140 is critical for resistance; this mutation is not present in the New Caledonia strains (Olesky, Hobbs et al. 2002). In contrast, a new mutation in the New Caledonia strains not previously recognized as one involved in resistance, A121S, is responsible for reduced susceptibility to penicillin. Additional mutations, however, are required for resistance to tetracycline, but these mutations have not yet been characterized.

One of the main differences between the -A and +T mutations in *mtrR* is the inability of the +T to activate mutations in $porb_{1b}$. It has been clearly shown that *penB*

mutations do not increase resistance to penicillin or tetracycline in the absence of a co-resident *mtrR* mutation (Olesky, Zhao et al. 2006). The mechanism of synergy between *mtr* and *penB* is unknown but presumably results in decreased influx of antibiotics. We were able to show that the -A mutation activated *penB* mutations in $porB_{1b}$ alleles from both FA6140 and strain 319, but conversely, the level of overexpression of the efflux pump provided by the +T mutation in strain 319 was not sufficient to activate the *penB* mutations. How strains in New Caledonia exhibit intermediate-levels of penicillin resistance without possessing an *mtrR* mutation capable of activating *penB* mutations is unclear. It is possible that they possess mutations in additional genes that are also capable of activating *penB* or that they utilize a completely different method of developing penicillin resistance.

Chapter 3.

Comparative overview of penicillin resistant and sensitive strains of *Neisseria gonorrhoeae*

3.1 Introduction

Though widespread penicillin resistance was first reported in *Neisseria gonorrhoeae* in 1987 and several groups (including ours) have studied the mechanisms of chromosomally-mediated β-lactam resistance, the mechanisms of high-level resistance are still not fully understood. The known resistance determinants (*penA, mtrR, penB, and ponA)* can be transferred from a resistant strain of *N. gonorrhoeae* (FA6140) to a sensitive strain (FA19) in the laboratory. Transformation with all of these determinants results in intermediate levels of resistance, but neither we nor others have been able to obtain transformants with donor-levels of resistance to penicillin. The inability to transform laboratory strains to donor levels of resistance has made elucidation of the mechanism of high-level resistance difficult. There are several possibilities to explain the inability to transform a recipient strain to the same level of resistance as the donor strain: 1) two or more genes are required to increase resistance; 2) resistance is conferred by a gene that is absent in recipient strains and therefore not readily transformable; or 3) the flanking sequences surrounding a gene are different in the two strains, thus preventing efficient recombination. In this chapter, we tested the hypothesis that resistant strains contain a gene that confers resistance, and that this gene is absent in recipient strains. By

identifying, cloning and transferring these genes to a recipient strain containing the four known resistance determinants (*penA*, *mtrR*, *penB*, and *ponA*; MD4), we hoped to gain insight into the genes that might contribute to high levels of resistance.

The recent advances in technology have made whole genome sequencing easier and more affordable, resulting in an increase in the availability of complete or partial genome sequences for many organisms. *Neisseria* species are capable of horizontal gene exchange and bioinformatics analyses have shown that commensal and pathogenic *Neisseria* share a large core genome (Marri, Paniscus et al. 2010). Beyond the core genome, many virulence genes are also shared between commensal and pathogenic species, though their roles in pathogenesis and virulence of the pathogens are not clear. In 2008-2009, the Broad Institute released drafts of the complete genome sequences of twelve strains of *N. gonorrhoeae*, allowing for more detailed studies and comparisons of different strains of the pathogens (Broad Institute). Among these strains were several isolates that our lab studies, including two penicillin-resistant strains (FA6140 and 35/02) and two penicillin-susceptible strains (FA19 and FA1090) (Table 3.1). A comparison of the strains revealed a set of 67 genes, expressed in 55 operons, that are found in the resistant strains but absent in the sensitive strains (Figure 3.1). These genes/operons were cloned into FA19 containing the four known determinants (MD4; FA19 *penA mtrR penB ponA*) to determine if any of these unique genes increases resistance to penicillin.

Figure 3.1 Relationship between strains of *N. gonorrhoeae*

The star indicates genes that are unique to resistant strains.

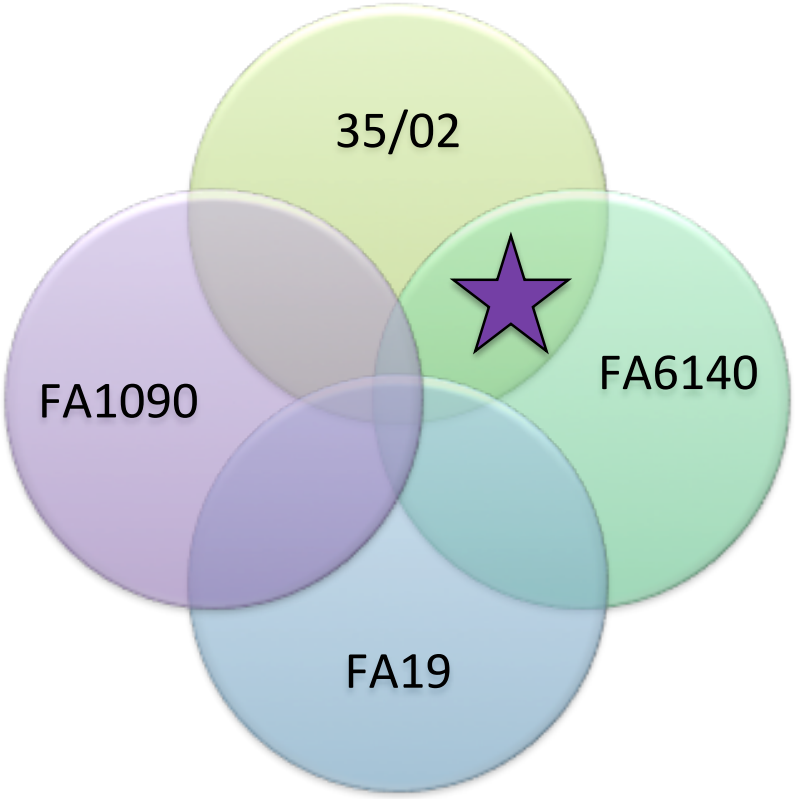


Table 3.1 Features of *N. gonorrhoeae* strains

Genomic contents of four strains of *N. gonorrhoeae* are shown below. FA19 and FA1090 are penicillin-susceptible strains while FA6140 and 35/02 are penicillin-resistant strains.

Strain	Size (nucleotides)	Number of open reading frames
FA19	2,099,891	2183
FA1090	2,153,922	2002
FA6140	2,033,960	2144
35/02	2,043,737	2131

3.2 Materials and Methods

3.2.1 Genome analysis

A reciprocal protein Basic Local Alignment Search Tool (BLAST) (Altschul, Madden et al. 1997) analysis comparing the proteomes of FA19 and FA1090 with FA6140 and 35/02 was carried out with assistance from the UNC Bioinformatics Core. Follow-up analysis was done in our lab. We performed a reciprocal nucleotide BLAST analysis (Altschul, Gish et al. 1990) to narrow down the results from the protein BLAST as sequencing errors could introduce false positives into the protein BLAST results. Candidate genes were selected from the nucleotide search and were defined as genes found in both FA6140 and 35/02 but not found in either FA19 or FA1090. Candidate genes are listed in Table 3.3. All genome information was obtained from the Broad Institute or from The National Center for Biotechnology Information (NCBI). BLAST searches were performed through the NCBI Basic BLAST website (NCBI), using standalone coding, or using CLC Genomics Workbench 5.0 Software (CLC Bio; Aahrus, Denmark).

3.2.2 Bacterial strains and plasmids

FA19 and FA6140 were described in Chapter 2. Strain 35/02 is a penicillin-resistant, cephalosporin intermediate-level resistant clinical isolate from Sweden, provided by Magnus Unemo of Örebro University Hospital, Örebro, Sweden. *N. gonorrhoeae* strain FA1090 is a well-characterized penicillin-sensitive clinical isolate used extensively in gonococcal studies (Nachamkin, Cannon et al. 1981; Hobbs, Sparling et al. 2011).

MD4 was created by transformation of FA19 with *penA*, *mtrR*, and *penB* PCR products using the transformation protocol described in Chapter 2. Penicillin was used for selection of *penA, penB,* and *ponA** transformants. Triton X-100 was used to select for *mtrR* transformants. Transformants were verified by sequencing. To transform *ponA** into MD3, a plasmid (pPR17) containing mutant *ponA* and an Ω cassette just downstream of the stop codon that confers resistance to spectinomycin and streptomycin was used. Positive transformants were selected for on GCB agar plates containing 100 μg/ml of spectinomycin. The Ω cassette was then removed by transforming again with PCR product of *ponA** and screening colonies for loss of spectinomycin resistance. Transformants were verified by genomic DNA amplification and sequencing.

Plasmid pKH35 was provided by Joseph Dillard from the University of Wisconsin at Madison. *E. coli* MC1061 cells were transformed with pKH35 containing each of the unique genes for transformation into MD4.

3.2.3 Preparation of DNA and PCR amplicands for genetic transformation

Primers were designed for each of the 55 operons listed in Table 3.3. Each 5' primer included a *PacI* restriction site and approximately 10 bases of upstream sequence before the start codon, while each 3' primer incorporated an *XbaI* restriction site following the stop codon to facilitate cloning into the pKH35 vector. Genomic DNA from FA6140 was extracted as described in Chapter 2. Genes were amplified from genomic FA6140 DNA using *Pfu* polymerase and the appropriate pairs primers. The PCR products were subcloned into the *PacI* and *XbaI* sites of the pKH35 vector and transformed into *E. coli* MC1061 cells. Because pKH35 contains a chloramphenicol resistance cassette, we

selected for positive transformants on LB agar plates containing 20 μg/mL chloramphenicol, and colonies appeared after incubation at 37 °C for 24-36 hours.

3.2.3 Transformation of *N. gonorrhoeae*

Despite repeated attempts, transformation of MD4 with pKH35 constructs was not possible, apparently due to MD4 having a lower transformation efficiency than FA19. As an alternative, we transformed each of the pKH35 constructs into FA19, following the same protocol described in Chapter 2. We selected for positive transformants on GCB agar plates with 0.2 μg/mL chloramphenicol, and these were verified by PCR amplification of boilates using pKH35 vector primers. Following verification of correct transformation, genomic DNA was prepared from each of the FA19-pKH35 strains and used to transform MD4. Chloramphenicol was again used for selection (0.6 μg/ml).

3.2.4 MIC determinations

The pKH35 vector puts genes under the control of an IPTG-inducible lac promoter. The plasmid recombines into the silent intergenic region between *lctP* and *aspC* (Stohl and Seifert 2001; Hamilton, Dominguez et al. 2005) (Figure 3.1). MICs were performed, following the same protocol described in Chapter 2. A second set of MICs were performed at the same time, with the addition of IPTG. Cells for the MICs with IPTG were grown the night before on GCB agar plates with 1mM IPTG. IPTG was also added to the antibiotic plates and added to the GCB+ broth used to dilute cells. Concentrations of penicillin between 0.125 and 1 .5 μg/mL were used to determine if the MIC of any of the new strains increased above that of MD4 (MIC = 0.7 μg/mL). MD4 was included as a control in every MIC experiment.

Figure 3.2 Graphic map of pKH35

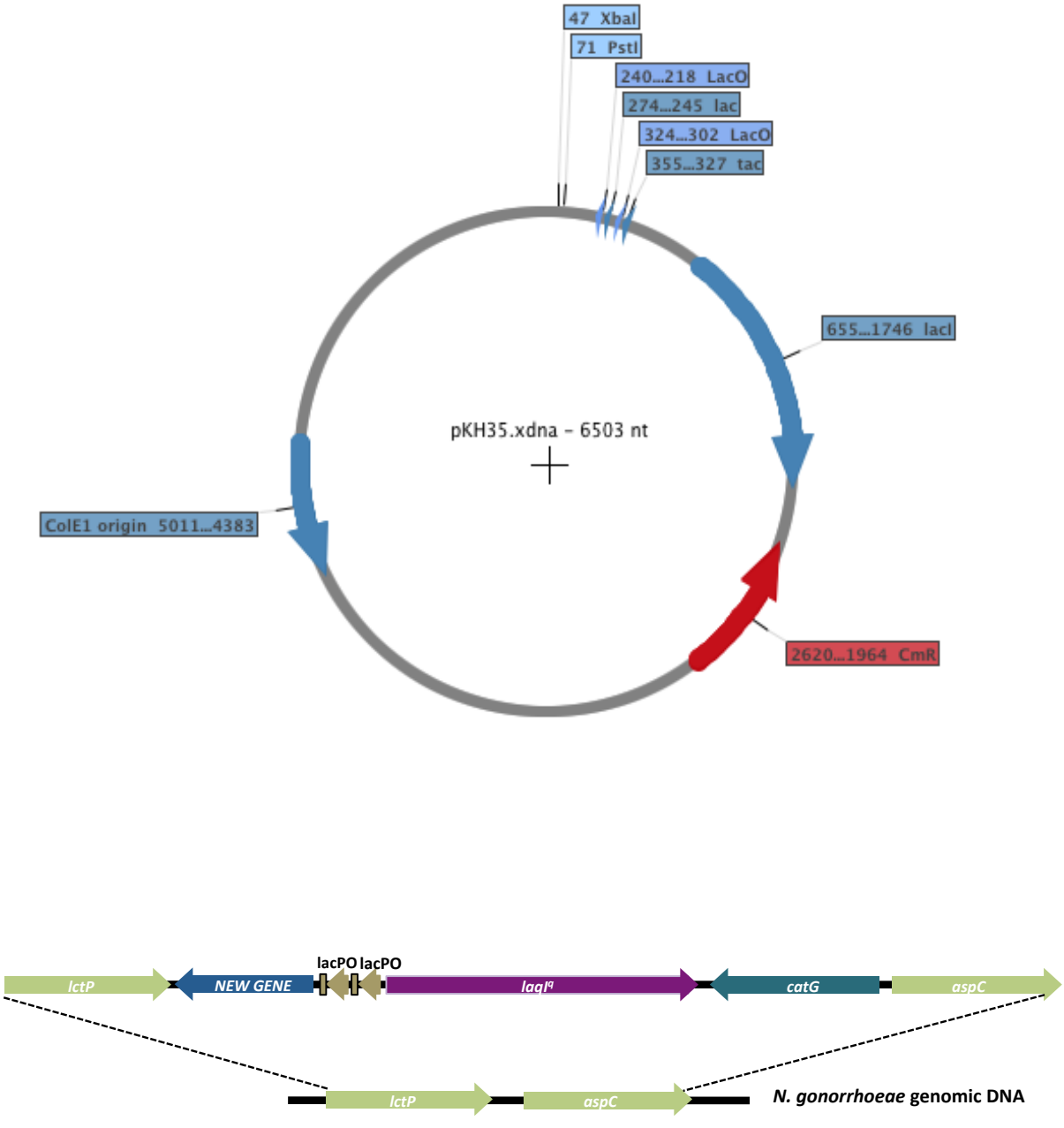


Table 3.2 Genes found only in penicillin resistant strains of *N. gonorrhoeae* (not in sensitive strains)

Gene number	Gene name	Size (nt)	Best BLAST hit (if any)
NGDG_0030	gene encoding conserved hypothetical protein	237	Histone-like nucleoid-structuring protein (GC-NCCP11945)
NGDG_0073	gene encoding predicted protein	201	
NGDG_0075	gene encoding predicted protein	165	
NGDG_0103	gene encoding predicted protein	174	
NGDG_0130	gene encoding predicted protein	186	gene encoding hypothetical protein (GC-NCCP11945)
NGDG_0164	gene encoding conserved hypothetical protein	186	gene encoding hypothetical protein (GC-NCCP11945)
NGDG_0197	gene encoding conserved hypothetical protein	186	gene encoding hypothetical protein (GC-TCDC-NG08107)
NGDG_0232	gene encoding predicted protein	165	gene encoding ferric enteric transporter binding protein (GC-NCCP11945)
NGDG_0299	gene encoding predicted protein	228	
NGDG_0362	gene encoding predicted protein	195	
NGDG_0428	gene encoding conserved hypothetical protein	420	*N. meningiditis* hypothetical protein
NGDG_0547	gene encoding predicted protein	306	gene encoding hypothetical integral membrane protein (GC-TCDC-NG08107)
NGDG_0676	gene encoding predicted protein	312	gene encoding putative phage associated protein (GC-TCDC-NG08107)
NGDG_0677	gene encoding conserved hypothetical protein	129	gene encoding hypothetical protein (GC-NCCP11945)

NGDG_0678	gene encoding predicted protein	408	gene encoding putative phage associated protein (GC-NCCP11945)
NGDG_0679	gene encoding conserved hypothetical protein	1161	gene encoding hypothetical protein (GC-NCCP11945, GC-TCDC-NG08107)
NGDG_0688	gene encoding predicted protein	306	
NGDG_0704	gene encoding predicted protein	198	
NGDG_0721	gene encoding predicted protein	345	gene encoding mercury transport protein (GC-NCCP11945)
NGDG_0739	gene encoding predicted protein	951	
NGDG_0762	gene encoding conserved hypothetical protein	1436	gene encoding hypothetical protein (GC-TCDC-NG08107)
NGDG_0784	gene encoding alcohol dehydrogenase class-III	1132	gene encoding alcohol dehydrogenase class-III (GC-NCCP11945, GC-TCDC-NG08107)
NGDG_0813	gene encoding conserved hypothetical protein	222	gene encoding HesB/YadR/YfhF family protein (GC-TCDC-NG0817)
NGDG_0816	gene encoding conserved hypothetical protein	495	gene encoding cell filamentation protein (GC-TCDC-NG0817)
NGDG_0823	gene encoding twin-arginine leader-binding protein DmsD	2219	gene encoding restriction modification protein Mod (GC-NCCP11945)
NGDG_0873	gene encoding lysR family transcriptional regulator	919	
NGDG_0874	gene encoding transposase	384	
NGDG_0876	gene encoding conserved hypothetical protein	603	gene encoding hypothetical protein (GC-TCDC-NG0817)

NGDG_0886	gene encoding hemagglutinin/hemolysin-related protein	5882	
NGDG_0887	gene encoding predicted protein	234	gene encoding hemagglutinin/hemolysin related protein
NGDG_0888	gene encoding conserved hypothetical protein	1851	gene encoding putative hemolysin activation protein HecB (GC-NCCP11945)
NGDG_0891	gene encoding conserved hypothetical protein	168	
NGDG_0904	gene encoding predicted protein	249	gene encoding putative phage associated protein (GC-TCDC-NG0817)
NGDG_0905	gene encoding conserved hypothetical protein	309	
NGDG_0906	gene encoding conserved hypothetical protein	1997	gene encoding bacteriophage tail protein keratin 2a (GC-TCDC-NG0817)
NGDG_0907	gene encoding predicted protein	228	gene encoding bacteriophage tail protein keratin 2a (GC-NCCP11945)
NGDG_0908	gene encoding predicted protein	1248	gene encoding polyprotein (GC-TCDC-NG0817)
NGDG_0911	gene encoding predicted protein	645	
NGDG_0915	gene encoding predicted protein	1280	
NGDG_0917	gene encoding predicted protein	159	gene encoding putative oxidoreductase (GC-TCDC-NG0817)
NGDG_1057	gene encoding predicted protein	162	
NGDG_1124	gene encoding conserved hypothetical protein	345	
NGDG_1258	gene encoding conserved hypothetical protein	135	gene encoding hypothetical protein (GC-NCCP11945)

NGDG_1267	gene encoding predicted protein	246	gene encoding glutamate dehydrogenase (GC-TCDC-NG0817)
NGDG_1296	gene encoding conserved hypothetical protein	366	gene encoding hypothetical protein (GC-TCDC-NG0817)
NGDG_1297	gene encoding conserved hypothetical protein	333	gene encoding hypothetical protein (GC-TCDC-NG0817)
NGDG_1298	gene encoding conserved hypothetical protein	372	gene encoding hypothetical protein (GC-TCDC-NG0817)
NGDG_1364	gene encoding predicted protein	204	gene encoding hypothetical protein (GC-NCCP11945)
NGDG_1371	gene encoding predicted protein	150	gene encoding pyridine nucleotide transhydrogenase (GC-TCDC-NG0817)
NGDG_1395	gene encoding transferrin-binding protein B	2097	partial tbpB gene for transferrin-binding protein B (GC strain 29528, GC-NCCP11945)
NGDG_1488	gene encoding conserved hypothetical protein	180	
NGDG_1490	gene encoding predicted protein	495	
NGDG_1497	gene encoding predicted protein	243	
NGDG_1511	gene encoding predicted protein	183	
NGDG_1595	gene encoding predicted protein	285	gene encoding hypothetical protein SdaA (GC-NCCP11945)
NGDG_1689	gene encoding outer membrane protein	708	gene encoding for *N. gonorrhoeae* outer membrane protein
NGDG_1700	gene encoding conserved hypothetical protein	117	gene encoding for hypothetical protein (GC-NCCP11945)

NGDG_1742	gene encoding conserved hypothetical protein	1530	
NGDG_1746	gene encoding transposase	645	**Homo sapien* DNA*
NGDG_1756	gene encoding predicted protein	264	gene encoding hypothetical protein (GC-NCCP11945)
NGDG_1864	gene encoding predicted protein	462	gene encoding hypothetical protein (*N. meningiditis* H44/76)
NGDG_1939	gene encoding predicted protein	153	gene encoding hypothetical protein (GC-TCDC-NG08107)
NGDG_1963	gene encoding conserved hypothetical protein	879	gene encoding putative phage associated protein (GC-NCCP11945)
NGDG_1978	gene encoding pilin	306	gene encoding pilS cassette (GC-NCCP11945)
NGDG_2013	gene encoding predicted protein	276	
NGDG_2014	gene encoding predicted protein	192	

3.3 Results

3.3.1 Results of BLAST searches

Original results from the protein BLAST search returned 217 proteins found only in the resistant strains of *N. gonorrhoeae*. Because errors are less likely to influence nucleotide BLAST searches than protein BLAST searches, the four genomes were compared again at the nucleotide level. If a gene is the same in all four strains, the gene from Strain A must be a "top hit" in a BLAST search of Strains B, C, and D. Similarly, when the same gene from Strain B is used as the query, it must be a top hit when Strains A, C, and D are searched. This pattern of reciprocal BLAST searching yielded 67 genes (Table 3.2) that met the following criteria: 1) The gene was a top hit in a reciprocal nucleotide BLAST search between FA6140 and 35/02. 2) The gene did not appear in a nucleotide BLAST search against FA19 or FA1090.

For subcloning into pKH35, I examined the locations of these genes in the FA6140 chromosome. Genes that were clustered together in what was likely the same regulon/operon were amplified as a unit, subcloned into pKH35, and transformed as one unit instead of as separate genes. This strategy was used because the genes would likely function as a unit, similar to the *mtrCDE* operon. The genes that were not part of an apparent operon were subcloned individually into pKH35 and transformed into MD4 as described in Materials and Methods.

3.3.2 Effects of the 55 genes/multigene clusters on resistance

Once all of the 55 genes/multigene clusters or operons were transformed into MD4, I performed MICs on all of the strains to determine any of these unique genes

contributed to resistance. Concentrations between 0.1 and 1.5 μg/mL were tested for each of the 55 transformants using a narrow dilution range (0.125, 0.25, 0.3, 0.4, 0.5, 0.6, 0.65, 0.7, 0.75, 0.8, 0.85, 0.9, 1.0, 1.25, 1.5 μg/mL), particularly around the MIC of MD4. Unfortunately, none of these genes had an effect on resistance to penicillin. The MIC of penicillin for MD4 is 0.7 μg/mL, and none of the strains had MICs above 0.7 μg/mL. Because we used a narrow dilution range, it was unlikely that changes in the MIC were missed because of widely spaced penicillin concentrations. The MIC experiments were also performed with and without 1 mM IPTG to induce the expression of the cloned genes/gene clusters, since the pKH plasmid puts the inserted genes under control of the IPTG-inducible lac promoter. Once again, we observed no differences in the MIC values with or without IPTG.

3.4 Discussion

Using a reciprocal BLAST search of the genome sequences from two penicillin-susceptible strains (FA19 and FA1090) and two penicillin-resistant strains (FA6140 and 35/02), we identified 67 unique genes shared by the two penicillin-resistant strains that were not in the genomes of the two penicillin-susceptible strains. These genes were cloned, transformed into a 4th level transformant (MD4), and their capacity to increase the MIC of penicillin up to the level of FA6140 or 35/02 was assessed. However, none of these genes or gen/clusters changed the MIC of penicillin in the transformants, indicating that none of these genes or clusters by themselves is involved in penicillin resistance.

Although the 67 genes were unique to FA6140 or 35/02, it is not clear if these genes were still capable of transforming FA19 from genomic DNA. Since the hallmark of "Factor X" and high-level resistance is the inability to be transferred by transformation, one of the prerequisites is that the genes would not readily transfer between the two sets of strains. However, since many of the genes are small in size and should have had enough flanking DNA to undergo homologous recombination with FA19, these genes could potentially be transformed even though they did not exist in the recipient strain. This scenario is also consistent with our data showing that transfer of these genes into MD4 using the pKH35 plasmid had no effect on penicillin resistance.

As shown in several studies, and discussed earlier in this dissertation, the *penB* mutation of porin is phenotypically silent without a co-resident mutation in *mtrR*. Similarly, the *ponA** mutation identified by our laboratory is found in many resistant clinical isolates, but inserting it into a strain containing the other known resistance determinants (FA19 *penA mtrR penB)* does not increase resistance any further, indicating

that expression of the *ponA** phenotype is dependent on other, unidentified factors (Ropp, Hu et al. 2002). It is possible that the 67 genes identified in this study also depend on an additional gene or mutation to be present in order to express a phenotye. In this case, we would not see an increase in the MIC by adding each gene individually, but would have to test every possible combination of the 67 genes, or the 55 operons. Because of the low likelihood of this method producing significant results, and because of the extraordinary amount of effort and time this would take, we elected to not pursue this approach.

There is still a possibility that might have seen a decrease in resistance in FA6140 if we had inactivated the genes and examined the effects of the inactivation on resistance. However, due to the amount of time and effort to make strains with inactivated genes, we elected not to continue with this approach. Instead, as detailed in Chapter 4, we investigated high-level resistance from a different angle, and these experiments provided significant new insight into this phenomenon.

The "Best BLAST Hit" of each of the 67 genes identified in the nucleotide BLAST is listed in Table 3.3; this table was updated at the time of writing this dissertation. Since the time when the original searches were performed, the two new *N. gonorrhoeae* genome sequences have been released with partial annotations: NCCP11945 and TCDC-NG08107 (Chung, Yoo et al. 2008; Chen, Hsia et al. 2011). At the time of our original bioinformatics studies, these sequences were not available and thus there were no matches to the genes in these strains. Interestingly, both of these strains are "multidrug resistant" strains of gonorrhea, though their MICs of penicillin have not been published. NCCP11945 was isolated in Korea while TCDC-NG08107 was isolated in Taiwan. If these strains exhibit high-levels of penicillin resistance, equivalent

or similar to those of FA6140 and 35/02, it would be interesting to include their genomic information in further studies.

The gene NGDG_1746 from *N. gonorrhoeae* strain FA6140 actually aligned to human chromosomal DNA. This was discovered only recently because previous BLAST searches had been limited to microbial organisms. This same sequence has been identified in other *N. gonorrhoeae* by the Seifert group (Anderson and Seifert 2011). They identified this gene as having 90-100% identity to human retrotransposable element L1 (LINE-1). In their studies, they showed a relatively low penetrance of this gene in gonococcal isolates, explaining its absence from FA19 and FA1090 in our analyses. The effects, if any, of the insertion of this foreign DNA into the chromosome are not likely associated with antibiotic resistance. However, this is a good example of the far reaching ability of *N. gonorrhoeae* to use horizontal gene exchange to acquire new genetic information for various reasons.

Chapter 4.

Characterization of the mechanism of high-level penicillin resistance

4.1 Introduction

Antibiotic resistance in the gonococci is a long-standing public health problem. As detailed in the introduction to this thesis, penicillin was really the first really effective antibiotic used to treat gonorrhea, and it remained so for 40 years. However, during that time, the MICs of penicillin for clinical isolates crept higher, until 1985, when an outbreak in the Durham bus station was found to be untreatable with penicillin (Faruki, Kohmescher et al. 1985). By 1987, penicillin had been removed as a treatment of choice by the Centers for Disease Control and Prevention. Other antibiotics, including tetracycline, azithromycin, and ciprofloxacin, also came and went on the approved treatment list, such that by 2007, only one class of antibiotics, the expanded-spectrum cephalosporins (in particular, ceftriaxone and cefixime) were the only antibiotics left on the recommended list. The appearance of strains with very high MICs for ceftriaxone does not bode well for future treatment of gonorrhea (Ohnishi, Golparian et al. 2011; Unemo, Golparian et al. 2012).

The mechanism of resistance to penicillin in the gonococci is complex and multi-faceted. Resistance can be transferred in a step-wise manner from a resistant donor strain to a susceptible recipient strain by DNA uptake and homologous recombination. These types of studies have identified four genes involved in penicillin resistance: *penA*

(mutations in PBP 2), *mtrR* (promoter mutation that increases expression of the MtrC-MtrD-MtrE efflux pump), *penB* (mutations in the $porB_{1B}$ allele that decrease antibiotic permeation into the periplasm, and *ponA* (mutation in PBP 1). Even when these four genes are transferred, the MIC of penicillin increases to 0.75 μg/ml, well below the donor level of resistance (MIC = 4 μg/ml). However, as detailed in this dissertation, transformation to higher levels of resistance has not been achieved, and this inability to identify the genetic mechanism of high-level resistance has markedly hindered our ability to understand the cellular mechanisms at play.

We have referred to the gene(s) involved in this last step of high-level resistance as "Factor X". As detailed in Chapter 3, there are several possibilities that explain the inability to transfer high-level resistance to susceptible strains by transformation: 1) two or more genes are required to increase resistance; 2) resistance is conferred by a gene that is absent in recipient strains and therefore not readily transformable; or 3) the flanking sequences surrounding a gene are different in the two strains, thus preventing efficient recombination. Studies in Chapter 3 investigated the possibility that resistance was due to a gene absent in the recipient strain, but the results from those studies indicated that such genes by themselves were not responsible for resistance.

Since transformation of Factor X is not possible, in this Chapter I initiated studies to learn more about the cellular mechanism of Factor X. Specifically, we asked 1) whether resistance mediated by Factor X was dependent on the known resistance determinants, and 2) whether Factor X resistance was specific for penicillin or other β-lactam antibiotics, or if it conferred resistance to other antibiotics as well. To address the first question, we created a series of step-wise revertants of FA6140 in which each of the

four known resistance determinants was reverted back to wild-type in a stepwise manner. This allowed for the comparison of known resistance determinants with and without the effect of Factor X at each level (Figure 4.1). To address the second question, we examined the MICs of a wide range of antibiotics, both bacteriostatic and bactericidal, for FA6140 and the highest level transformant of MD4 for each particular antibiotic. These studies revealed new insight into how Factor X increases resistance.

4.2 Materials and Methods

4.2.1 Bacterial strains and plasmids

Strains FA19 and FA6140 have been described earlier in this dissertation. Strains MD1, MD2, MD3, and Strain MD4 were constructed by step-wise transformation of FA19 with PCR products of each of the four known resistance determinants amplified from FA6140, as described in Chapter 3. In all cases, correct transformation was verified by PCR amplification of genomic DNA and sequencing.

Revertant strains of FA6140 were created by replacing each of the four known resistance determinants in a step-wise manner with its corresponding wild-type allele. To replace *ponA** with the wild-type *ponA* gene, a plasmid (pPR16^{WT}; Figure 4.1) containing wild-type *ponA* and an Ω cassette just downstream of the stop codon that confers resistance to spectinomycin and streptomycin was used to transform FA6140. Positive transformants (referred to hereafter as MDrev3) were selected on GCB agar plates containing 100 μg/mL spectinomycin (Ropp, Hu et al. 2002). The next determinant, *penB*, was reverted to wild-type PorB$_{1B}$ by transforming MDrev1 with pBScat-1090PIB, which contains the *porB$_{1b}$* gene from FA1090, a kanamycin resistance cassette just past

the stop codon, and ~500 bp of flanking sequence on both ends of the gene (Figure 4.1) (Olesky, Hobbs et al. 2002). Transformants (referred to as MDrev2) were selected on GCB agar plates with 40 μg/mL kanamycin.

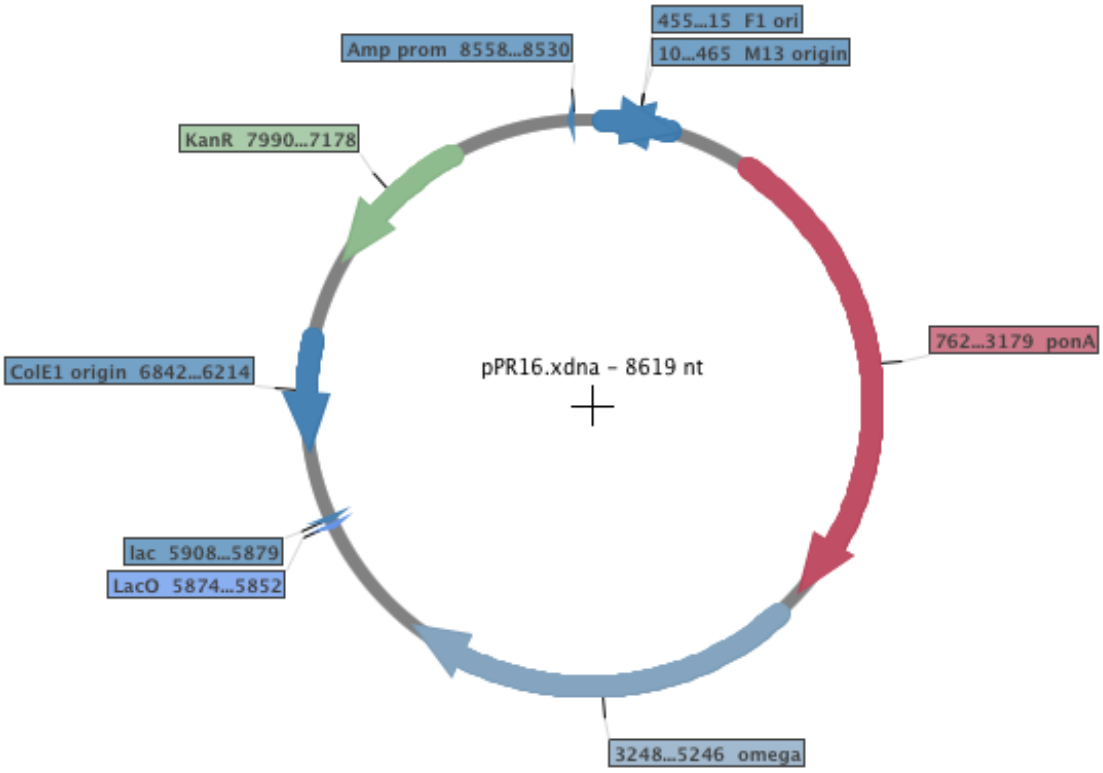
Reverting the last two determinants, *mtrR* and *penA*, back to wild-type was not as straight-forward as there was no selection marker for transformants. To revert *mtrR* back to wild-type, FA19 genomic DNA was amplified with primers flanking the *mtr* promoter region, and transformation was performed according to our standard laboratory protocol as described earlier. Because of the lack of selection available, a reverse selection method was used to identify correct transformants. The transformation mixture was diluted and plated on plain GCB agar plates to obtain individual colonies. The following day, approximately 250 colonies were streaked onto GCB plates with or without 8 μg/mL Triton X-100. Colonies that grew on Triton X-100 plates after 24 hours were considered to be negative (i.e. they still harbored the *mtrR* mutation from FA6140). Colonies that did not grow on Triton X-100 plates but did grow on plain GCB plates contained the wild-type *mtrR* promoter sequence.

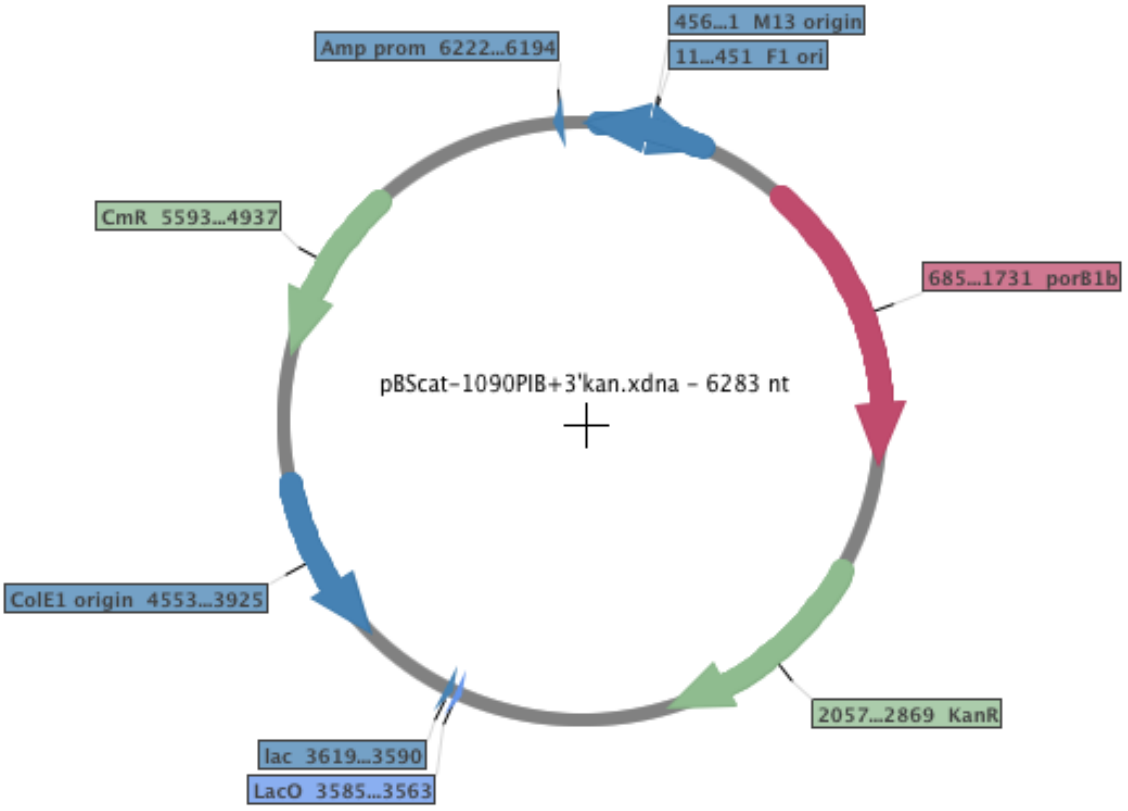
A plasmid, puc18us-*penA* (courtesy of Joshua Tomberg) was used to in the final step in the reversion of FA6140. This plasmid contains the wild-type *penA* gene from FA19 and the *N. gonorrhoeae* uptake sequence to facilitate transformation and homologous recombination. The QuikChange Method (Stratagene; Carlsbad, CA) was used to incorporate silent restriction sites into the *penA* gene (Tomberg, Unemo et al. 2010), which were utilized to screen for correct transformants (Figure 4.1). MDrev3 (FA6140 with wt *ponA, penB, mtr*) was transformed with puc18us-*penA* and plated on plain GCB plates. The next day, approximately 300 colonies were passaged onto GCB

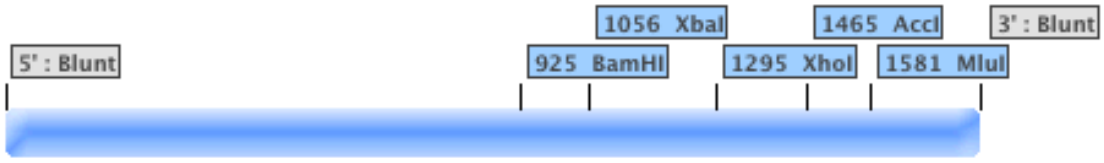
agar plates containing 0.25 μg/mL penicillin and onto plain GCB agar plates. Colonies that did not grow on the penicillin plates but did grow on the plain plates were considered positive for the wild-type *penA* gene. Boilates were prepared from positive transformants and the *penA* gene was amplified by PCR. To verify that the wild-type *penA* gene had recombined into the genome, the PCR products were digested with *BamHI* and *XhoI* (New England Biolabs; Ipswich, MA), which are two of silent restriction sites in the *penA* clone. Clones that appeared positive by digest were verified by sequencing.

Figure 4.1 Plasmids used to generate FA6140 reversion strains

pPR16 was used to transform wt-*ponA* into FA6140. pBScat-1090PIB was used to transform wt-*porB* into MDrev1 (FA6140-wtponA). FA19 *penA* with silent restriction sites is shown in the third panel.







FA19 penA + Silent RE sites - 1746 nt

Table 4.1 Strains and plasmids used in this study

Strain	Description
FA19	Pen and tet sensitive laboratory strain
FA6140	CMRNG clinical isolate
FA19-pIB	FA19 transformed with pBScat-1090PIB+3'kan
MD1-pIB	FA19pIB transformed with FA6140 *penA* PCR product
MD2-pIB	MD1-pIB transformed with FA6140 *mtrR* PCR product
MD1	FA19 transformed with FA6140 *penA* PCR product
MD2	FA19 transformed with FA6140 *penA* PCR product
MD3	FA19 transformed with FA6140 *penA*, *mtrR*, *penB* PCR products
MD4	MD3 transformed with FA6140 *ponA** PCR product
MDrev3	FA6140 transformed with pPR16 (wt-*ponA)*
MDrev2	MDrev1 transformed with pBScat-1090PIB+3'kan
MDrev1	MDrev2 transformed with FA19 *mtrR* PCR product
MDrev0	MDrev3 transformed with puc18us-FA19*penA*
pPR16	plasmid containing wild-type FA19 *ponA* and Ω fragment for streptomycin/spectinomycin resistance
pBScat-1090PIB+3'kan	plasmid containing FA1090 $porB_{1b}$ allele and kanamycin resistance cassette
puc18us-$penA^{wt}$	plasmid containing GC uptake sequence and FA19*penA* with silent restriction sites throughout *penA* coding sequence

4.2.2 MIC determinations

The MICs of a range of antibiotics for the two series of strains in FA19 (MD1-MD4) or FA6140 (MDrev1-MDrev4) containing each of the different resistance determinants were determined as described in Chapter 2.

4.2.3 Transformation of MD4

When examining resistance to different antibiotics, it was necessary to transform MD4 with FA6140 DNA to be sure that there were no additional resistance determinants (other than *penA*, *mtrR*, *penB*, and *ponA*) in FA6140 that provided increased resistance to the antibiotic. We followed the genomic transformation protocol described in Chapter 2 to transform MD4 to the highest levels of resistance for each antibiotic tested in these studies. Briefly, piliated MD4 cells were incubated with genomic DNA extracted from FA6140. After five hours, the transformation mixture was plated on a concentration of the appropriate antibiotic slightly higher than the MIC (determined previously). Plates were checked after 24 and 48 hours for the presence of colonies.

4.2.4 Bactericidal killing curves

Gonococcal cells were grown in liquid medium and time-kill measurements after the addition of various antibiotics were performed as previously described (Crenn, Meyran et al. 1994). Briefly, GCB broth was inoculated with *N. gonorrhoeae* cells at a density of 10^8 CFU/mL. Cultures were incubated in a shaking incubator at 37 °C for approximately one hour, or until $OD_{560} = 0.25\text{-}0.3$. At this point, cultures were re-diluted to 10^8 CFU/mL and allowed to grow for two hours. After two hours, a 1mL aliquot was

removed and diluted to OD_{560} = 0.18. Aliquots were plated on GCB agar plates and incubated at 37 °C. Antibiotics at concentrations twice the MICs were added to flasks containing aliquots of the original liquid culture. One flask without antibiotic was used as a control. Flasks were kept in the shaking incubator and samples were removed and plated on GCB agar at 1, 2, and 4 h. Colonies were counted after 24 hours and the cfu/mL were calculated.

Experiments involving thiourea and 2,2'-dipyridyl (Sigma-Aldrich; St. Louis, MO) were identical to the antibiotic killing curves. Either thiourea or 2,2'-dipyridyl were added to flasks with the antibiotics at the same time. *N. gonorrhoeae* was grown with thiourea and with 2,2'-dipyridyl to ensure that the compounds did not negatively affect bacterial growth at the concentrations used.

4.3 Results

4.3.1 The phenotype of Factor X is expressed independently of other resistance determinants

To determine whether the phenotype of Factor X was dependent on any of the known resistance determinants, each of the known determinants in FA6140 was reverted back to wild-type in a step-wise manner and the MICs of the revertant strains were compared to strains of FA19 containing the same known resistance determinants (Figure 4.2). Thus, the only difference in resistance determinants between the strains being compared (e.g. MD3 vs MDrev3 or MD2 vs MDrev2) is Factor X.

As shown in Figure 4.3, the MIC of penicillin was 2-4 fold higher in each of the FA6140 revertant strains compared to the corresponding FA19 strains, indicating that the phenotype of Factor X for conferring penicillin resistance is expressed independently of the other determinants. Likewise, the MICs of chloramphenicol, ciprofloxacin, tetracycline and rifampin for the revertant strains were also consistently 2- to 4-fold higher than the corresponding FA19 strains containing the same known determinants. In contrast, the MIC of erythromycin was equivalent for the FA19 and FA6140 strains lacking *mtrR* mutations (Figure 4.3).

Figure 4.2 Stepwise transfer and reversion of chromosomally mediated resistance genes

The left side of this figure shows the FA19 series of strains that have an increasing number of resistance determinants. MD1 has only the first determinant, *penA*. MD2 has the first two determinants, *penA* and *mtr*. MD3 and MD4 have *penB* and *ponA* added, respectively. The right side of the figure depicts a series of strains in which each of the known determinants in FA6140 was reverted back to wild-type in a step-wise manner. The nomenclature is the same as for the FA19 series: The number in the strain name indicates how many known resistance determinants are present. For example, MDrev3 is the FA6140 parental strain with the *ponA*-wild-type allele, the three remaining determinants (*penA*, *mtr*, and *penB*) are still present.

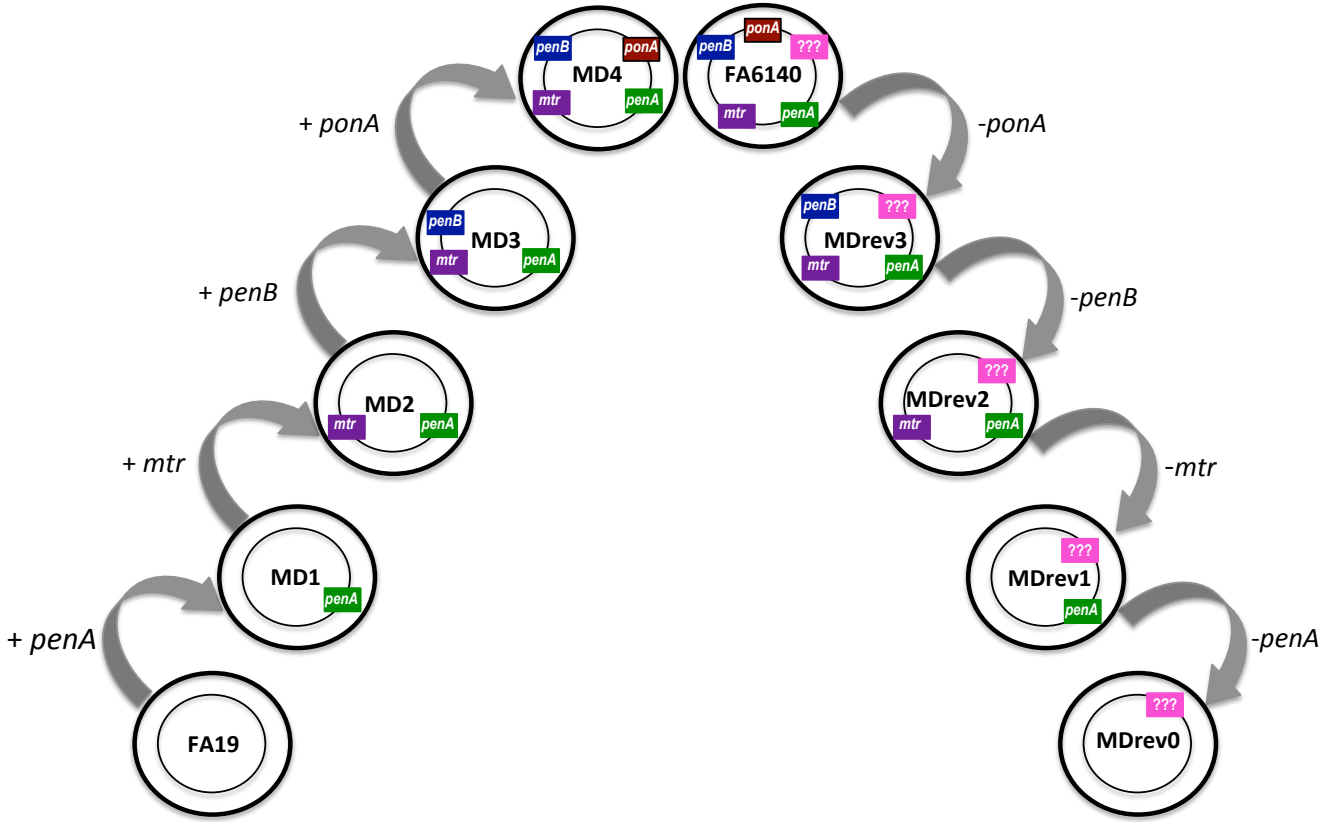
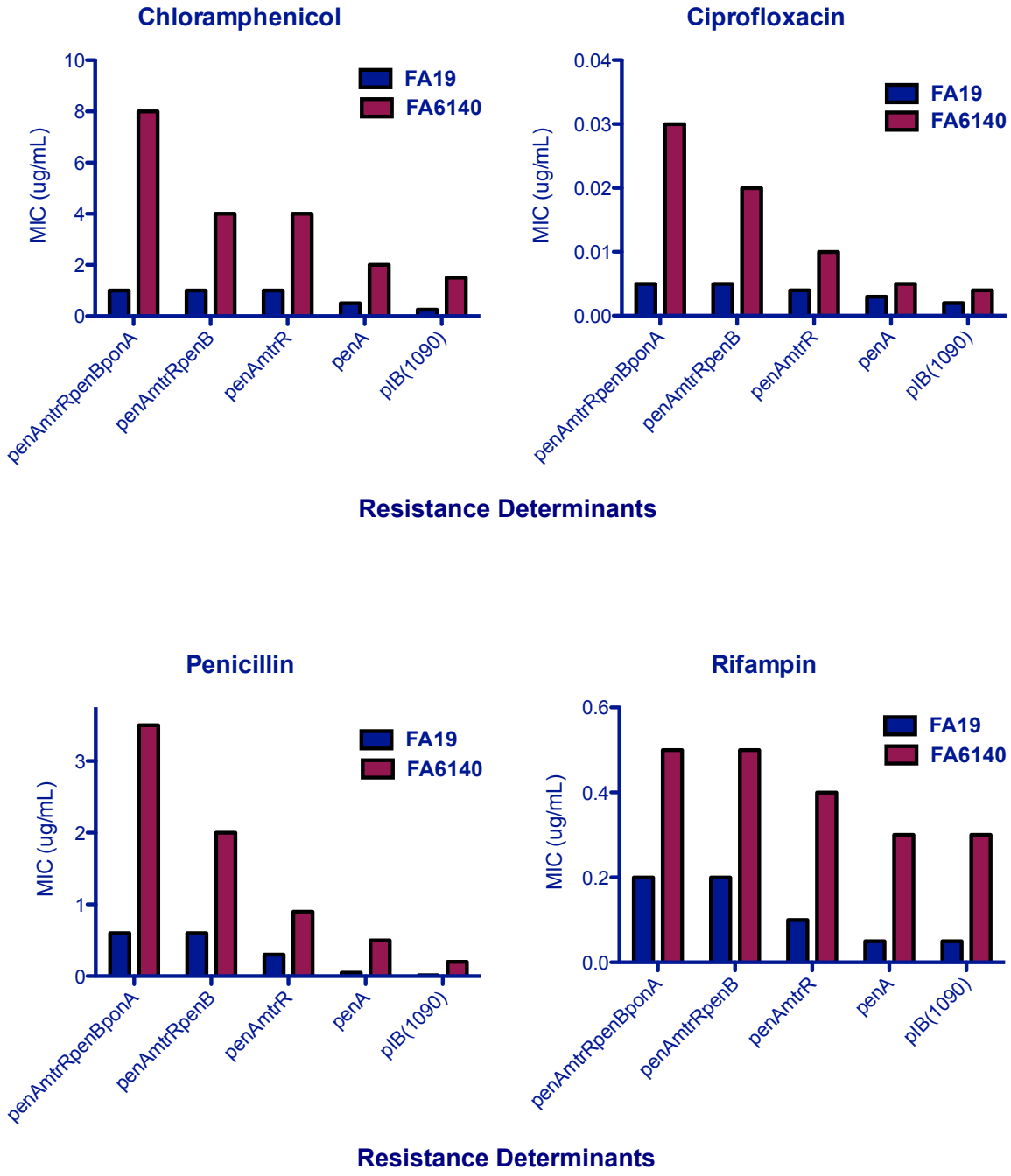
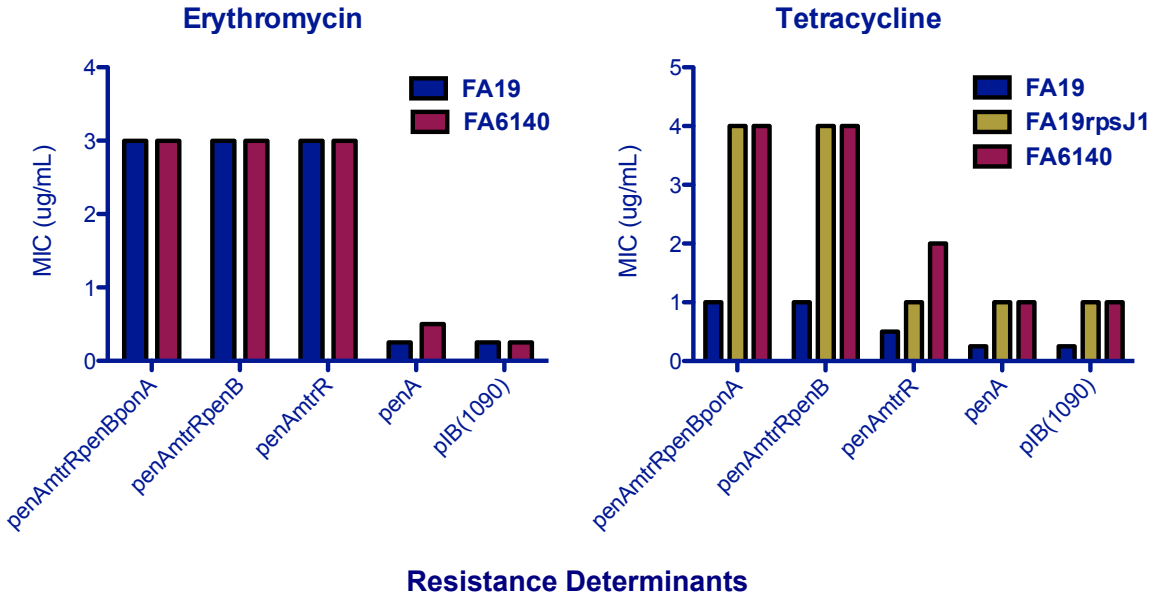


Figure 4.3 MICs of paired FA19 transformants and FA6140 revertants

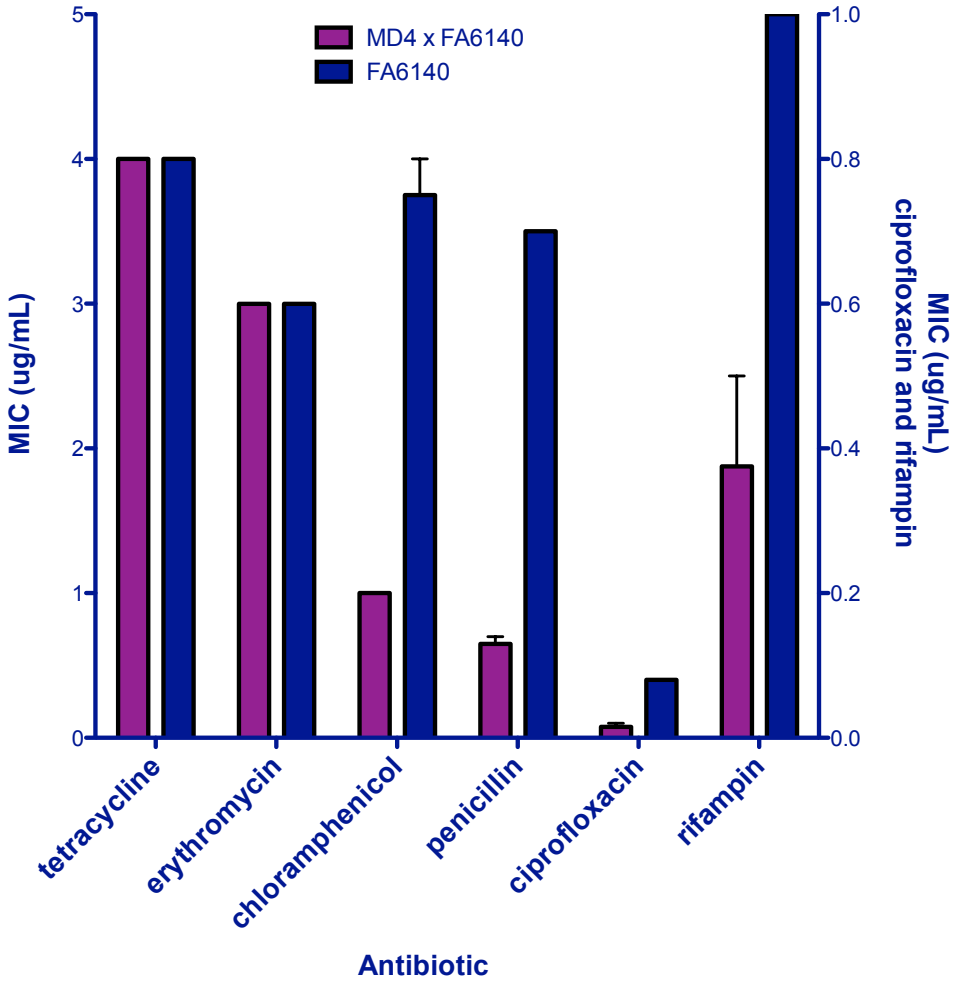



Erythromycin
MIC (ug/mL)
0
1
2
3
4
FA19
FA6140
penAmtrRpenBponA
penAmtrRpenB
penAmtrR
penA
pIB(1090)
Tetracycline
MIC (ug/mL)
0
1
2
3
4
5
FA19
FA19rpsJ1
FA6140
penAmtrRpenBponA
penAmtrRpenB
penAmtrR
penA
pIB(1090)
Resistance Determinants

4.3.2 Factor X influences the MICs of some, but not all, antibiotics

To ensure that differences were not due to any other transformable mutations, MD4 was transformed with genomic DNA from FA6140 and transformants were selected on concentrations of the antibiotic just above the MIC for MD4. For example, we have shown that there is an additional resistance determinant for tetracycline, *rpsJ1*, which encodes an altered S10 ribosomal protein (Hu, Nandi et al. 2005). As shown in Figure 4.3, when the *rpsJ1* allele from FA6140 was introduced into MD4, the MIC of tetracycline was equivalent to the donor strain, FA6140. In contrast to tetracycline, we were unable to obtain transformants for the antibiotics chloramphenicol, ciprofloxacin, penicillin, or rifampin. Thus, as shown in Figure 4.4, donor levels of resistance were obtained with the highest transformant of MD4 (MD4 x FA6140 gDNA) for erythromycin and tetracycline, but not with chloramphenicol, penicillin, ciprofloxacin, and rifampin.

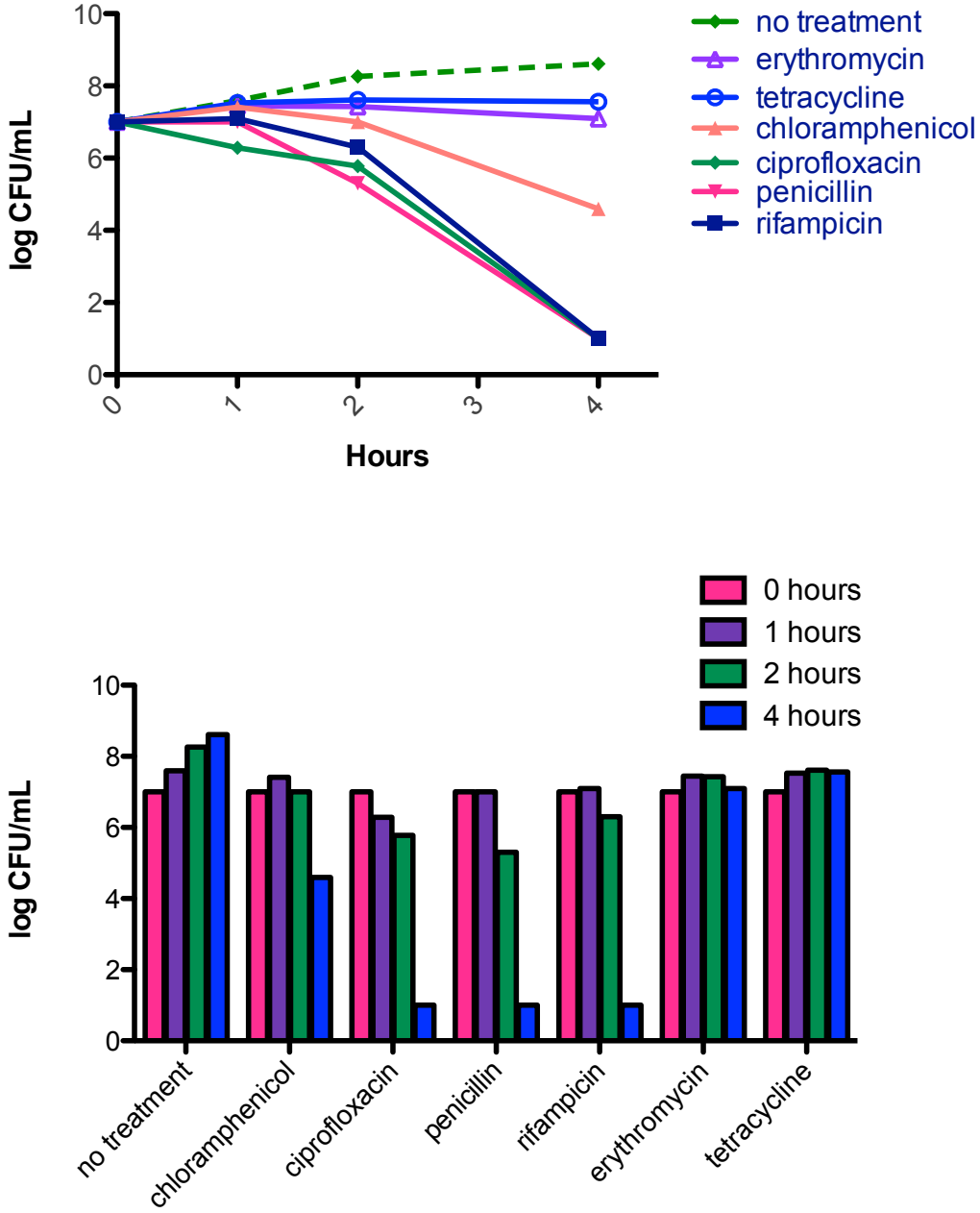
Figure 4.4 MICs of transformants of MD4 with FA6140 DNA



4.3.3 Effects of various antibiotics on the growth of *N. gonorrhoeae* in liquid culture

The results from Figure 4.4 indicate that two antibiotics, erythromycin and tetracycline, have the same MIC for both FA6140 and the highest transformant of MD4. These two antibiotics are usually bacteriostatic, whereas most of the antibiotics that do not have the same MICs for the two strains are generally considered to be bactericidal. To determine their mode of action in *N. gonorrhoeae*, we diluted log-phase liquid cultures of FA6140 to $OD_{560} = 0.1$, added the different antibiotics at twice their MIC, and determined the cfu/mL for the cultures every hour for 4 hours. As shown in Figure 4.5, cells with no antibiotic continued to grow, as expected. Cultures treated with erythromycin or tetracycline were arrested in growth but did not decrease the cfu/mL, indicating that these drugs are bacteriostatic for *N. gonorrhoeae*. In contrast, chloramphenicol, ciprofloxacin, penicillin, and rifampicin all killed the cells as shown by the decrease in cfu/mL of the treated cultures, demonstrating that they are bactericidal for *N. gonorrhoeae*.

Figure 4.5 Time-kill curves of *N. gonorrhoeae* treated with various antibiotics



4.3.4 Effects of iron chelators and hydroxyl radical quenchers on survival of *N. gonorrhoeae* treated with antibiotics in liquid culture

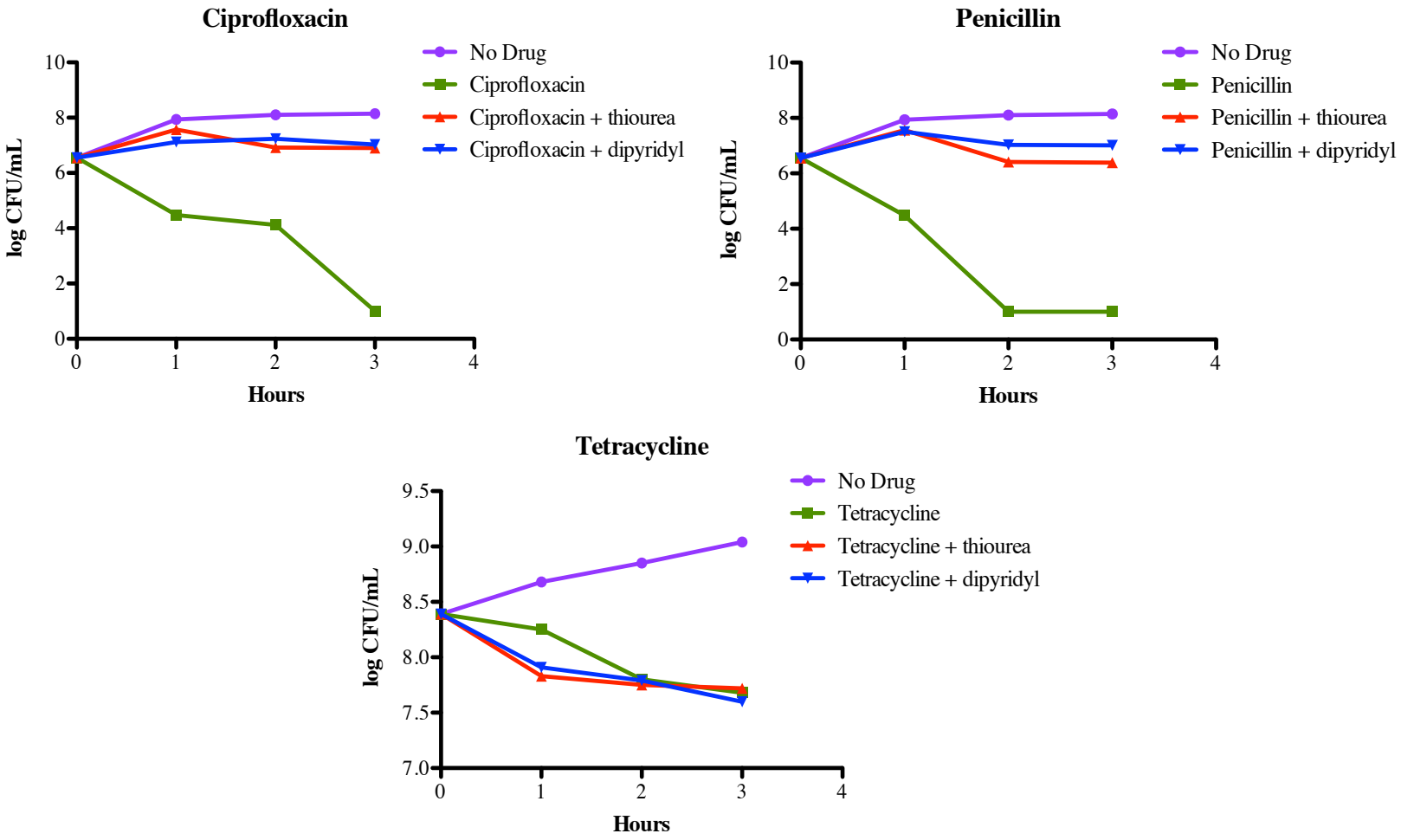
The data from the MIC, transformation, and kill-curve experiments indicate that Factor X confers non-specific resistance to bactericidal antibiotics but has no effect on bacteriostatic drugs. These data are highly reminiscent of recent studies in *E. coli* showing that bactericidal (but not bacteriostatic) antibiotics kill by a common mechanism of action involving the production of hydroxyl radicals ($OH^{\bullet}$) and metabolic stress (Kohanski, DePristo et al. 2010). Thus, no matter the pathway bactericidal antibiotics inhibit, they all kill the bacteria at concentrations near their MIC by a common metabolic stress mechanism.

To investigate whether the formation of $OH^{\bullet}$ was involved in bactericidal antibiotic killing of *N. gonorrhoeae*, we performed kill-curve assays in the presence thiourea, a selective $OH^{\bullet}$ scavenger (Repine, Fox et al. 1981; Novogrodsky, Ravid et al. 1982; Wasil, Halliwell et al. 1987). As shown in Figure 4.6, the addition of thiourea prevented the killing of FA6140 by two bactericidal antibiotics with different mechanisms of action, penicillin and ciprofloxacin, whereas thiourea had no effect on the bacteria treated with the bacteriostatic antibiotic, tetracycline.

Hydroxyl radicals are formed when an electron is transferred from ferrous iron (Fe^{2+}) to hydrogen peroxide through the Fenton reaction (Imlay 2003). To demonstrate that the production of $OH^{\bullet}$ upon the addition of bactericidal antibiotics was a result of the Fenton reaction, we performed kill curve experiments with the same antibiotics as above in the presence of the iron chelator, 2,2'-dipyridyl, which inhibits the Fenton reaction by chelating free iron within the cell (Imlay, Chin et al. 1988). Similar to our results with thiourea, 2,2'-dipyridyl protected *N. gonorrhoeae* from killing by the bactericidal

antibiotics, penicillin and ciprofloxacin, but had no significant effect on the bacteria treated with the bacteriostatic antibiotic, tetracycline (Figure 4.6).

Figure 4.6 Effects of thiourea and 2,2'-dipyridyl on growth of *N. gonorrhoeae* treated with antibiotics



4.4 Discussion

When we started the experiments outlined in this chapter, we knew very little about the characteristics of Factor X, outside of the fact that high-level resistance to penicillin was not transferrable by homologous recombination. We asked whether Factor X required any of the other known determinants to exert its resistance phenotype, and whether it was specific for β-lactam antibiotics or also mediated resistance to other antibiotics. The surprising results indicated that Factor X provides higher resistance to all bactericidal antibiotics and does not depend on other resistance determinants. Finally, we showed that *N. gonorrhoeae* is killed by bactericidal antibiotics by initiation of an oxidative stress mechanism that involves released Fe^{2+} and formation of hydroxyl radicals.

To provide corroborating evidence that bactericidal antibiotics kill *N. gonorrhoeae* by release of Fe^{2+} and formation of hydroxyl radicals, we treated liquid cultures of *N. gonorrhoeae with* either thiourea (a selective hydroxyl radical scavenger) or 2,2'-dipyridyl (an iron scavenger), and both of these compounds protected gonococci from killing by bactericidal antibiotics. These data are similar to results reported by Kohanski, *et al*., who showed that bactericidal antibiotics induce cell death of *E. coli* through the production of hydroxyl radicals ($OH^{\bullet}$). Hydroxyl radicals are deadly to bacterial cells, causing damage mainly to DNA, but also to membranes, proteins, and lipids (Imlay 2003).

Many groups have studied the oxidative stress defense mechanisms of *N. gonorrhoeae*, but the mechanisms are quite different from those of *E. coli*, which are much better understood. *N. gonorrhoeae* is exposed to reactive oxygen species (ROS)

produced by commensal bacteria in its host environment, ROS from human immune system defenses, and ROS and reactive nitrogen species (RNS) as byproducts of its own metabolic processes. Many genes have been identified as important either for protection from or in response to oxidative damage. For example, catalase and peroxidase are important for detoxifying H_2O_2 and Mn^{2+} quenches $O_2^{\bullet-}$ (Seib, Wu et al. 2006). However, bacteria do not have a defined mechanism for destroying $OH^{\bullet}$ (Dwyer, Kohanski et al. 2009). Studies in *E. coli* showed that treatment with fluoroquinolones resulted in up-regulation of genes involved in $O_2^{\bullet-}$ stress, iron-sulfur cluster synthesis, and iron uptake. $O_2^{\bullet-}$ levels in the cell are increased by increased metabolic activity, such that the normal cellular defenses against $O_2^{\bullet-}$ damage are overwhelmed. Ferric iron (Fe^{3+}) is reduced to ferrous iron $(Fe^{2})^{+}$ in the Haber-Weiss reaction, and Fe^{2+} is then free to oxidize H_2O_2 through the Fenton reaction to form $OH^{\bullet}$, which kills the bacterial cells (Dwyer, Kohanski et al. 2007; Dwyer, Kohanski et al. 2009) (Figure 4.7).

Superoxide and hydroxyl radicals were first implicated in cellular death caused by fluoroquinolones. Further studies showed that all bactericidal antibiotics, regardless of their target, also caused death through generation of damaging hydroxyl radicals. This common mechanism of cell death has been reported in *E. coli* and also in *Staphylococcus aureus* (Kohanski, Dwyer et al. 2007). Although further studies are needed, we can tentatively add *N. gonorrhoeae* to the list of bacteria in which bactericidal antibioitcs cause death through hydroxyl radical formation.

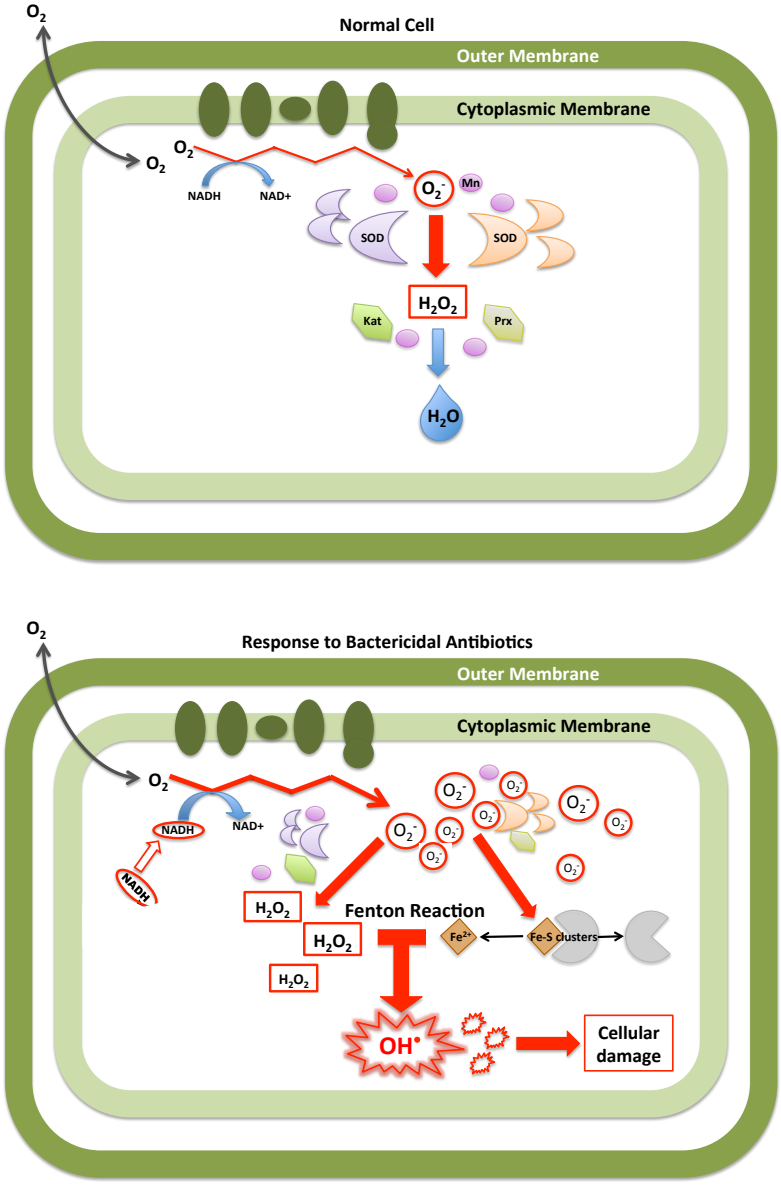
Though the studies described here do not directly demonstrate an antibiotic-dependent increase in $OH^{\bullet}$ formation in *N. gonorrhoeae*, our results do show a direct decrease in bactericidal antibiotic killing when cultures were protected from toxic effects

of $OH^{\bullet}$. We also showed that the bacterium responds to the two different classes of antibiotics differently, and that Factor X increases resistance non-specifically to bactericidal antibiotics but does not have an effect on bacteriostatic antibiotics. We were unable to transform to high levels of resistance for all bactericidal antibiotics tested, and the resistant strain with all resistance determinants reverted to wild-type had a 2- to 4-fold higher MIC than the sensitive strain for all bactericidal drugs.

Based on these observations, we propose a mechanism for Factor X related to oxidative stress defenses in the pathogen: Resistant strains, such as FA6140, have developed mechanisms of protecting against oxidative stress more efficiently than sensitive strains, such as FA19. Since all bactericidal agents, independent of the target, kill by producing ROS and thus damaging DNA, proteins, and membranes, resistant strains of *N. gonorrhoeae* likely change the regulation of some set of genes involved in DNA repair, cell wall synthesis, or iron regulation and a higher concentration of antibiotic is necessary for killing. Because this is not a single, or even double, gene event, it has been impossible to transform to levels of high resistance in the laboratory. Studies to identify which genes are regulated in response to bactericidal antibiotics in *N. gonorrhoeae* will be introduced Chapter 5.

Figure 4.7 Proposed model of oxidative stress response in *N. gonorrhoeae*

Under normal conditions (top panel) $O_2^{\bullet-}$ is produced during respiration and in response to the environment. Defenses include superoxide dismutase (SOD) and (Mn), which in turn produce H_2O_2. Catalases (kat) and peroxidases (Prx) detoxify peroxide. When treated by bactericidal antibiotics (bottom panel), increased metabolism and respiration and cellular responses result in an increase in $O_2^{\bullet-}$, overwhelming the oxidative stress defenses. Ferrous iron is released and reacts with H_2O_2 to produce $OH^{\bullet}$. Figure adapted from (Seib, Wu et al. 2006; Dwyer, Kohanski et al. 2009)



Chapter 5.

Conclusions and future directions

5.1 Transcriptome sequencing of N. gonorrhoeae

5.1.1 Introduction

The data I have presented in this thesis has centered on understanding the genetic, biochemical, and mechanistic aspects of high-level penicillin resistance in the pathogenic organism, *N. gonorrhoeae*. We established that the bacterium responds differently to bactericidal and bacteriostatic antibiotics and that Factor X is involved only in resistance to bactericidal drugs. We were able to transform a sensitive strain to levels of donor resistance for bacteriostatic drugs, but could not reach the same levels of resistance of bactericidal drugs. Furthermore, we reverted each of the known resistance determinants in a resistant strain (FA6140) back to wild type and observed that the phenotype of Factor X was independent of any known determinants.

Multiple studies have shown that all bactericidal drugs kill *E. coli* through the production of hydroxyl radicals. When normal cellular defense mechanisms are overwhelmed, excess oxidants build up within the bacterial cell. Hydroxyl radicals are formed through the interaction of excess hydrogen peroxide and reduced iron in the Fenton reaction. We have also shown that in *N. gonorrhoeae*, all bactericidal drugs also kill by producing hydroxyl radicals and that the effects of these drugs can be blocked by the addition of substances that inhibit the Fenton reaction (thiourea or 2,2'-bipyridyl).

These results led us to examine the role of oxidative and metabolic stress pathways in the -cidal action of the antibiotics, and our data support a role of hydroxyl radical formation in this process. Based on our results, we hypothesize that FA6140 does not initiate the metabolic stress response until higher levels of antibiotic are present compared to FA19 and FA1090, and that this difference is due to up-regulation or higher expression of important proteins that help delay the metabolic stress response. To better understand the gonococcal response to antibiotic treatment, we sequenced the transcriptomes of FA6140 and MD4 (FA19 *penA mtrR penB ponA**) under normal and penicillin-stressed conditions, allowing us to compare the transcriptomes of a penicillin-resistant and a penicillin-sensitive strain under normal and penicillin-stressed conditions. The experiments and data described here represent our first attempts to understand the transcriptome of FA6140 compared to MD4 and to glean from these data potential mechanisms that underlie the resistance phenotype of FA6140.

5.1.2 Materials and Methods

Liquid cultures of *N. gonorrhoeae* were grown as described above. Upon reaching log phase, MD4 and FA6140 cultures were each divided into two 250 mL baffled flasks. One flask of each strain was treated with penicillin. MD4 was treated with 0.175 μg/mL and FA6140 was treated with 1 μg/mL of penicillin for 1 hr; these concentrations were selected from earlier trials because they inhibited the growth of cells, indicating that they were placing stress on the cells without killing them. Cells were harvested from 5 ml of culture at 3000 x *g* for 5 min at 20ºC and resuspended in 3ml RNA later (Ambion; Austin, TX). Following incubation at 4ºC overnight, cells were treated with 10 mM Tris-

HCl, 1 mM EDTA (TE buffer) plus 0.2 mg/mL lysozyme. The SpinSmart RNA Purification kit (Denville Scientific; Metuchen, NJ) was used to extract RNA according to manufacturer instructions. After purification, the RNase inhibitor RNasin Plus (Promega; Madison, WI) was used to preserve RNA integrity and the RQ1 DNase kit (Promega) was used to remove residual DNA contamination. Both the quality and quantity of RNA were determined by A_{260} and $A_{260.280}$ ratio respectively. The MicrobExpress bacterial mRNA enrichment kit (Ambion) was used to remove 16S and 23S rRNA by following the manufacturer's protocol. The UNC High Throughput Sequencing Facility performed two rounds of transcriptome sequencing using the Illumina HiSeq 2000 and the Illumina Genome Analyzer II (Illumina; San Diego, CA).

5.1.3 RNA-seq results

We used two alignment methods to map reads from the sequencing data to the *N. gonorrhoeae* genome sequence. Because FA1090 is well annotated in NCBI, it was chosen as the primary reference genome for aligning sequencing reads. Galaxy is a public server that allows users with minimal programming experience to run computational analyses on large datasets (Giardine, Riemer et al. 2005; Blankenberg, Von Kuster et al. 2010; Goecks, Nekrutenko et al. 2010). CLC Genomics Workbench 5.0 software was used for additional analysis (CLC Bio; Aahrus, Denmark). Once reads were aligned to FA1090, comparisons were made between penicillin-treated and -untreated samples of MD4 and FA6140 to determine changes in gene expression caused by antibiotic treatment. We were most interested in genes with a 2-fold or greater change in expression, by reads per kilobase of transcript per million-mapped-reads (RPKM).

Interestingly, more genes had increased expression in MD4 than FA6140 when treated with penicillin (36 vs. 15) (Table 5.1). These results led to the hypothesis that FA6140 might express higher levels of oxidative stress response genes under normal conditions than MD4 does. If this were the case, there might not be a difference in gene expression upon addition of penicillin or other bactericidal antibiotics, because the bacterium is already primed and ready for attack by the antibiotic. To test this, we compared the transcriptomes of MD4 and FA6140. This comparison showed 274 genes that have higher basal levels of expression in FA6140 while only 56 are expressed at higher levels in MD4 (Table 5.2).

Many of the genes expressed at higher levels in FA6140 encode for proteins that might help to protect the cell from oxidative stress and antibiotic attack. For example, several proteins are involved in DNA repair mechanisms or cell wall/membrane biogenesis. Hydroxyl radicals induce death by damaging DNA, membranes, and proteins. With the upregulation of genes involved in repairing and regenerating these essential cellular components, FA6140 might be more capable of protecting itself from hydroxyl radical mediated damage than is MD4.

Table 5.1 Genes with increased expression in FA6140 in response to penicillin treatment

NGO0228	Hypothetical protein	other/unknown
NGO0492	phage associated protein	other/unknown
NGO0797	transcriptional regulator	transcription
NGO1095	phage associated protein	other/unknown
NGO1115	phage associated protein	other/unknown
NGO1136	hypothetical protein	cell wall/membrane biogenesis
NGO1208	NgoMIIIM	restriction endonuclease
NGO1209	DNA-cytosine methyltransferase	replication, recombination, repair
NGO1313	hypothetical protein	other/unknown
NGO1623	phage associated protein	other/unknown
NGO1633	phage associated protein	other/unknown
NGO1634	phage associated protein	other/unknown
NGO1635	phage associated protein	other/unknown
NGO1636	phage associated protein	replication, recombination, repair
NGO1739	hypothetical protein	replication, recombination, repair

Table 5.2 Genes with increased expression in MD4 in response to penicillin treatment

fxsA	phage T7 exclusion suppressor	other/unknown
hslO	Hsp33-like chaperonin	posttranslational modification
NGO0228	hypothetical protein	other/unknown
NGO0375	phosphoglucomutase	carbohydrate transport/metabolism
NGO0376	peptidyl-prolyl cis-trans isomerase B	posttranslational modification
NGO0457	hypothetical protein	other/unknown
NGO0475	phage associated protein	other/unknown
NGO0476	phage associated protein	other/unknown
NGO0477	phage associated protein	transcription
NGO0478	phage associated protein	other/unknown
NGO0479	lambda repressor protein	transcription
NGO0487	phage associated protein	other/unknown
NGO0488	phage associated protein	other/unknown
NGO0554	hypothetical protein	other/unknown
NGO0854	hypothetical protein	other/unknown
NGO0879	hypothetical protein	other/unknown
NGO1014	phage associated protein	Transcription
NGO1015	phage associated protein	transcription
NGO1046	ClpB protein	posttranslational modification
NGO1095	phage associated protein	other/unknown
NGO1096	phage associated protein	other/unknown
NGO1105	phage associated protein	other/unknown
NGO1115	phage associated protein	other/unknown
NGO1208	NgoMIIIM	restriction endonuclease
NGO1209	DNA-cytosine methyltransferase	replication, recombination, repair
NGO1244	MarR family transcriptional regulator	transcription
NGO1422	heat shock protein	posttranslational modification
NGO1496	transferrin-binding protein B	other/unknown
NGO1564	hypothetical protein	other/unknown
NGO1633	phage associated protein	other/unknown
NGO1634	phage associated protein	other/unknown
NGO1770	oligopeptidase A	other/unknown

Table 5.3 Genes with increased basal levels of expression in FA6140 compared to MD4

Genes are classified according to predicted function by COG (Clusters of Orthologous Groups) analysis (Natale, Galperin et al. 2000; Tatusov, Galperin et al. 2000).

Replication, recombination, and repair	
NGO0007	putative type II restriction endonuclease
NGO0300	putative very-short-patch-repair endonuclease
NGO0485	putative replicative DNA helicase, putative phage associated protein
NGO0545	putative type III restriction-modification system methyltransferase
NGO0738	putative DNA-damage-inducibile protein
NGO0771	putative exodeoxyribonuclease V alpha subunit
NGO0973	putative DNA polymerase III, epsilon chain
NGO1074	MafI protein 2
NGO1146	putative phage associated protein
NGO1157	IS1016 transposase
NGO1256	IS1016 transposase
NGO1259	DNA topoisomerase IV subunit A
NGO1412	IS1016 transposase
NGO1437	primosome assembly protein PriA
NGO1648	Irg7
NGO1795	DcmB
NGO1907	hypothetical protein
NGO1987	hypothetical protein
radC	
rdgC	
recX	
Amino acid transport and metabolism	
avtA	valine-pyruvate transamidase
NGO0444	threonine dehydratase
NGO0532	hypothetical protein
NGO0835	GNA1162
NGO1182	putative nitrogen regulatory protein P-II
NGO1241	histidinol-phosphate aminotransferase
NGO1329	aromatic amino acid aminotransferase
NGO1600	glutamine synthetase
NGO1773	hypothetical protein

NGO1808	D-amino acid dehydrogenase small subunit
NGO2013	putative ABC transporter, ATP-binding protein

Posttranslational modification and protein turnover

anmK	anhydro-N-acetylmuramic acid kinase
NGO0132	NrdG protein
NGO1372	cb-type cytochrome c oxidase subunit IV
NGO1656	hypothetical protein NGO1656
smpB	SsrA-binding protein

Coenzyme transport and metabolism

bioD	dithiobiotin synthetase
hemC	porphobilinogen deaminase
NGO0360	hypothetical protein
NGO0857	putative dihydroneopterin aldolase
NGO1307	DNA/pantothenate metabolism flavoprotein
NGO1483	8-amino-7-oxononanoate synthase
NGO1508	pyridoxal phosphate biosynthetic protein

Translation

def	peptide deformylase
NGO0208	hypothetical protein
NGO0549	hypothetical protein
NGO1330	tRNA (uracil-5-)-methyltransferase
NGO1783	RluC
NGO1831	50S ribosomal protein L29
NGO1842	elongation factor Tu
NGO1870	methionyl-tRNA formyltransferase
rplA	50S ribosomal protein L1
rplL	50S ribosomal protein L7/L12
rpsB	30S ribosomal protein S2
rpsL	30S ribosomal protein S12
truB	tRNA pseudouridine synthase B

Carbohydrate transport and metabolism

eno	phosphopyruvate hydratase
gmhA	phosphoheptose isomerase
NGO0142	putative sugar transporter
NGO1911	hypothetical protein
NGO2076	putative glucokinase
pgi	glucose-6-phosphate isomerase

pgk	phosphoglycerate kinase
ppnK	inorganic polyphosphate/ATP-NAD kinase
Energy production and conversion	
gltA	type II citrate synthase
NGO0143	hypothetical protein
NGO0651	hypothetical oxidoreductase
NGO0734	putative oxidoreductase
NGO0890	D-lactate dehydrogenase
NGO0893	putative oxidoreductase
NGO0923	putative succinate dehydrogenase flavoprotein subunit
NGO0984	hypothetical protein
NGO1024	hypothetical protein
NGO1026	putative ferredoxin
NGO1279	nitroreductase
NGO1413	Na(+)-translocating NADH-quinone reductase subunit A
NGO1414	Na(+)-translocating NADH-quinone reductase subunit B
NGO1417	Na(+)-translocating NADH-quinone reductase subunit E
NGO1442	ethanol-active dehydrogenase/acetaldehyde-active reductase
NGO1521	putative acetate kinase
NGO1525	methylcitrate synthase
NGO1741	NADH:ubiquinone oxidoreductase subunit K
NGO1743	NADH dehydrogenase subunit I
NGO1751	NADH:ubiquinone oxidoreductase subunit A
NGO1775	putative ferredoxin
NGO2031	PetC
Nucleotide transport and metabolism	
hemH	phosphoribosylaminoimidazole-succinocarboxamide synthase
NGO0589	putative ABC transporter, permease protein
NGO0748	putative phosphoribosylaminoimidazole carboxylase catalytic subunit
NGO1183	phosphoribosylformylglycinamidine synthase
NGO1876	aspartate carbamoyltransferase regulatory subunit
NGO2009	putative permease protein

nrdB	ribonucleotide-diphosphate reductase subunit beta
Cell wall/membrane biogenesis	
lnt	apolipoprotein N-acyltransferase
lolB	outer membrane lipoprotein LolB
mraY	phospho-N-acetylmuramoyl-pentapeptide-transferase
NGO0205	LolA
NGO0344	GTP-binding protein LepA
NGO0418	putative glycosyl transferase
NGO0534	alginate O-acetylation - like protein
NGO0626	putative murein hydrolase
NGO0815	putative UDP-N-acetylmuramate: L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase
NGO0834	hypothetical protein NGO0834
NGO0987	ADP-heptose--LPS heptosyltransferase II
NGO1216	diacylglycerol kinase
NGO1801	hypothetical protein
Cell cycle control and mitosis	
maf	Maf-like protein
NGO0653	hypothetical protein
NGO1659	hypothetical protein
Transcription	
NGO0007	putative type II restriction endonuclease
NGO0288	RNA polymerase factor sigma-32
NGO0801	putative ribonuclease
NGO1407	AsnC family transcriptional regulator
NGO1850	DNA-directed RNA polymerase subunit beta'
Inorganic ion transport and metabolism	
NGO0363	putative stress-sensitive restriction system protein
NGO0542	hypothetical protein
NGO1195	EriC protein
NGO1433	putative ABC transporter, permease protein
NGO1581	putative transport protein
NGO1769	CcpR
NGO2088	putative ABC transporter, ATP-binding protein, enterobactin
NGO2090	putative ABC transporter, permease protein

trkA	potassium transporter peripheral membrane component
Signal transduction mechanisms	
NGO0176	putative two-component system sensor kinase
NGO0541	putative phage associated protein
NGO1064	hypothetical protein
NGO1298	hypothetical protein
NGO1382	putative GTP pyrophosphokinase
NGO1866	nitrogen assimilation regulatory protein NtrX
NGO0181	putative sec-independent protein translocase component
NGO0184	HitA
NGO1156	hypothetical protein
Defense mechanisms	
NGO0237	N-acetyl-anhydromuranmyl-L-alanine amidase
NGO0395	multidrug efflux protein
NGO0407	putative type I site-specific deoxyribonuclease
NGO0546	putative type III restriction-modification system endonuclease protein
NGO0186	putative zinc-binding alcohol dehydrogenase
NGO1249	alkylhydroperoxidase
NGO1879	putative bacteroicin resistance protein
Other/undefined	
NGO0033	hypothetical lipoprotein
NGO0039	hypothetical protein
NGO0046	hypothetical protein
NGO0049	hypothetical protein
NGO0054	hypothetical protein
NGO0055	pilus-associated protein
NGO0121	hypothetical protein
NGO0130	hypothetical protein
NGO0163	hypothetical protein
NGO0232	hypothetical protein
NGO0252	hypothetical lipoprotein
NGO0302	hypothetical protein
NGO0315	hypothetical protein
NGO0342	hypothetical protein
NGO0357	hypothetical protein
NGO0377	putative transport protein

NGO0391	hypothetical protein
NGO0422	hypothetical protein
NGO0454	hypothetical protein
NGO0455	hypothetical protein
NGO0461	hypothetical protein
NGO0465	putative phage associated protein
NGO0472	putative phage associated protein
NGO0474	putative phage associated protein
NGO0482	putative phage associated protein
NGO0484	putative phage associated protein
NGO0510	putative phage associated protein
NGO0511	hypothetical protein
NGO0527	hypothetical protein
NGO0613	hypothetical protein
NGO0640	RmsR
NGO0666	hypothetical protein
NGO0678	hypothetical lipoprotein
NGO0709	hypothetical protein
NGO0729	putative phage associated protein
NGO0746	putative O-methyltransferase
NGO0780	hypothetical protein
NGO0791	hypothetical protein
NGO0811	hypothetical protein
NGO0842	hypothetical protein
NGO0843	hypothetical protein
NGO0919	hypothetical protein
NGO0927	hypothetical protein
NGO0937	hypothetical protein
NGO1005	putative phage associated protein
NGO1010	putative phage associated protein
NGO1011	putative phage associated protein
NGO1025	hypothetical protein
NGO1037	hypothetical protein
NGO1062	hypothetical protein
NGO1107	putative phage associated protein
NGO1121	putative phage associated protein
NGO1130	putative phage associated protein
NGO1139	putative phage associated protein
NGO1150	hypothetical protein
NGO1166	putative phage associated protein

NGO1215	hypothetical protein
NGO1257	hypothetical protein
NGO1293	hypothetical protein
NGO1306	hypothetical protein
NGO1346	hypothetical protein
NGO1348	hypothetical protein
NGO1352	3-oxoacyl (acyl-carrier protein) synthase
NGO1370	integral membrane protein
NGO1385	hypothetical protein
NGO1386	hypothetical protein
NGO1388	hypothetical protein
NGO1449	LctP
NGO1459	hypothetical protein
NGO1463	hypothetical protein
NGO1491	integral membrane protein
NGO1503	hypothetical protein
NGO1584	MafB3
NGO1585	MafB4
NGO1586	hypothetical protein
NGO1591	hypothetical protein
NGO1593	hypothetical protein
NGO1595	hypothetical protein
NGO1597	hypothetical protein
NGO1620	putative phage associated protein
NGO1629	putative phage associated protein
NGO1630	putative phage repressor protein
NGO1642	putative phage associated protein
NGO1643	putative phage associated protein
NGO1645	putative phage associated protein
NGO1647	putative phage associated protein
NGO1681	hypothetical protein
NGO1692	hypothetical protein
NGO1719	hypothetical protein
NGO1726	hypothetical protein
NGO1753	hypothetical protein
NGO1760	hypothetical protein
NGO1847	hypothetical protein
NGO1849	hypothetical protein
NGO1861	hypothetical protein
NGO1865	DNA processing protein

NGO1872	hypothetical protein
NGO1874	hypothetical protein
NGO1880	hypothetical protein
NGO1931	GapC
NGO1938	hypothetical protein
NGO1955	hypothetical protein
NGO2004	hypothetical protein
NGO2010	hypothetical protein
NGO2016	hypothetical protein
NGO2017	hypothetical protein
NGO2047	hypothetical lipoprotein
NGO2075	homoserine kinase
NGO2086	hypothetical protein
NGO2113	hypothetical protein
NGO2118	outer membrane transport protein
NGO2120	NTP binding protein
NGO2123	hypothetical protein
NGO2125	putative acyltransferase
NGO2135	putative transglycosylase
NGO2137	putative ABC transporter
NGO2168	3-oxoacyl-(acyl carrier protein) synthase III
NGO2170	hypothetical protein

5.1.4 Discussion and future directions

We are currently testing the hypothesis that penicillin-resistant strains of *N. gonorrhoeae,* such as FA6140, are able to more efficiently handle damage caused by oxidative stress than penicillin-sensitive strains, such as MD4 and FA19. The results of the transcriptome analysis indicated that FA6140 expresses many genes involved in protection from oxidative stress as well as genes that might assist the bacterium with repairing damage caused by oxidative stress. To determine if the increased expression of these genes is responsible for the increased resilience of FA6140 to antibiotic killing, we plan on cloning these genes into a gonococcal expression and targeting vector to overexpress them in MD4. If they do indeed induce a protective effect, the MIC of penicillin will increase when these genes are expressed at higher levels in MD4.

Our studies have demonstrated a clear role for hydroxyl radical-mediated killing of *N. gonorrhoeae* by bactericidal antibiotics. We have shown that different strains, such as FA6140 and MD4, have very different responses to the same antibiotics. To further understand these differences, we have obtained plasmids from Dr. Ann Jerse at Uniformed Services University of the Health Sciences to knock-out several oxidative stress response genes. We will utilize these plasmids to knock-out catalase (*kat*), cytochrome C peroxidase (*ccp*), the manganese transporter (*mntC*), and methionine sulfoxide reductase (*msrA*) in both FA6140 and MD4. These genes are known be important for detoxifying oxidants that cause damage to *N. gonorrhoeae* cells, but their roles in protection against attack by antibiotics, if any, have not yet been tested. We hypothesize that since bactericidal antibiotics kill *N. gonorrhoeae* through oxidative stress mechanisms and the production of hydroxyl radicals, these genes will confer

protection against bactericidal antibiotics and conversely, when these genes are absent, the MICs of these antibiotics will drop. Because FA6140 might have higher expression levels of other genes that protect against oxidative stress, the MICs of bactericidal antibiotics might not decrease for this strain upon the knock-out of *kat*, *sodB*, or *gor*.

5.2 General discussion and conclusions

N. gonorrhoeae has evolved with man and been an important pathogen for thousands of years. The bacterium has developed mechanisms to evade the human immune system, allowing it to successfully avoid killing and establish infections that can cause serious complications for the host. Gonorrhea infections are most often localized to the genital tract, but if left untreated, can lead to pelvic inflammatory disease, ectopic pregnancy, and infertility in women, as well as disseminated gonococcal infections in both sexes. The ability of *N. gonorrhoeae* to develop antibiotic resistance to antibiotics makes treating gonococcal infections difficult.

Two mechanisms of antibiotic resistance have been identified in the gonococcus. Plasmid-mediated resistance involves the acquisition of a plasmid harboring a gene that inactivates the antibiotic. This mechanism of resistance has been identified for both penicillin and tetracycline. Chromosomally mediated resistance, which is more common and more complicated, involves the step-wise acquisition of several resistance determinants. Chromosomally mediated resistance to penicillin has been studied extensively but is still not fully understood. Four determinants that contribute to intermediate levels of resistance have been identified. 1) *penA,* which encodes for an altered form of penicillin-binding protein 2 (PBP 2), the primary target of penicillin; 2)

mtrR, which is most often a mutation in the promoter of the MtrC-MtrD-MtrE efflux pump, resulting in its overexpression; 3) *penB*, which encodes for mutations in the major outer membrane porin that decrease influx of antibiotics into the periplasm; and 4) *ponA*, which encodes a mutated form of PBP 1, the other essential PBP in *N. gonorrhoeae*. Transfer of these four determinants from a resistant strain to a susceptible strain in the laboratory is possible and results in intermediate levels of resistance. However, despite multiple attempts by several labs, including ours, transformation to levels of resistance equivalent to the donor strain has not been possible. The goals of my studies were to develop a deeper understanding of the known determinants *mtrR* and *penB* and also to characterize high-level penicillin resistance.

I characterized unique *mtrR* and *penB* mutations identified in a group of *N. gonorrhoeae* strains isolated in New Caledonia with intermediate levels of penicillin resistance. In studies described in Chapter 2, I showed that the New Caledonia *mtrR* and *penB* determinants had much weaker phenotypes than the determinants from FA6140, a well characterized penicillin-resistant strain. *penB* mutations are dependent on a co-resident *mtrR* mutation (Olesky, Zhao et al. 2006), but the unique mutation in the New Caledonia *mtrR* determinant was unable to activate *penB* mutations, indicating that another mechanism is involved in resistance in these strains.

In chapter 3, I used bioinformatics tools to analyze and compare the genome sequences of resistant and sensitive strains of *N. gonorrhoeae*. Reciprocal protein and nucleotide BLAST analyses demonstrated that there were 67 genes, found in 55 transcriptional units, found exclusively in penicillin-resistant strains of the bacterium. To determine if these genes contributed to resistance, I transformed each of them into MD4,

a penicillin-sensitive strain containing the four known resistant determinants. I performed MIC experiments on each of the transformed strains, but results showed that none of these genes increased resistance to penicillin.

Studies in Chapter 4 were designed to further characterize the cellular mechanisms behind high-level penicillin resistance. Because of the inability to transform to donor levels of penicillin resistance in the lab, identification of "Factor X", or the gene(s) causing high levels of resistance has not been possible. Experiments to learn more about how Factor X mediates high-level resistance were designed and carried out. A series of step-wise revertants of FA6140 was created, in which each of the four known resistance determinants was reverted back to wild type in a step-wise manner. By comparing these strains with a series of strains containing the same resistance determinants transformed into a sensitive strain, I was able to demonstrate that the phenotype of Factor X is independent of any of the four known resistance determinants. I next determined the MICs of FA6140 and the highest-level transformant possible for several antibiotics. The results showed that MD4 could be transformed to the levels of resistance for FA6140 for all of the bacteriostatic antibiotics tested, but I was unable to reach full donor levels of resistance for any of the bactericidal antibiotics. Finally, I showed that *N. gonorrhoeae* is killed by bactericidal antibiotics through an oxidative stress mechanism that involves damage to the cell by the formation of hydroxyl radicals. To better understand why FA6140 is able to better fight off attack by bactericidal antibiotics, I have initiated ongoing studies in our lab that are based on results of transcriptome analysis of FA6140 and MD4, both with and without penicillin stress.

My original search to define "Factor X" as a single gene that contributes to high-level penicillin resistance in *N. gonorrhoeae* developed into a hypothesis that high-level resistance might not be caused by a single mutated gene at all. Results from the transcriptome analysis of MD4 and FA6140 show a striking difference in gene expression under normal circumstances. FA6140 expresses many genes at much higher levels, including several genes that have known functions in protecting against oxidative damage and aiding the bacterium in repairing the cell wall and DNA. Combined with the results from Chapter 4 demonstrating that bactericidal antibiotics kill *N. gonorrhoeae* through the production of hydroxyl radicals and oxidative stress mechanisms, this gene expression data provide a compelling argument for the hypothesis that FA6140, and other resistant strains, have evolved mechanisms to protect themselves from attack by bactericidal antibiotics.

The problem of antibiotic resistance in *N. gonorrhoeae* is ongoing, and the development of resistance to every class of antibiotics used to treat gonococcal infections makes this an important public health issue. There is emerging resistance to ceftriaxone and cefixime, the current drugs used to treat gonorrhea and fears that gonorrhea may become untreatable are not unfounded. The findings described in this dissertation and the studies that are planned for the future will help to develop our knowledge about how resistance develops and how the pathogen responds to antibiotics, hopefully aiding in the design of new therapeutics.

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